Effect of sampling on coagulation variables and effect of submaximal physical exercise on ADVIA™2120 platelet activation indices, platelet function, secondary and tertiary hemostasis as well as thrombelastography in healthy dogs

ELIF ER

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eingereicht von

Elif Er

Tierärztin aus Darmstadt

Gießen 2012

Mit Genehmigung des Fachbereichs Veterinärmedizin
der Justus-Liebig-Universität Gießen

Dekan: Prof. Dr. Dr. h. c. Martin Kramer

Gutachter:
Prof. Dr. Andreas Moritz
Prof. Dr. Joachim Roth

Tag der Disputation:
03.05.2012

For my dear grandfather Şükrü Er

(Sevgili dedem Şükrü Er'e ithaf edilmiştir)

INDEX

Abbrevations

α_1-PI	α_1 protease inhibitor
α_2-AP	α_2-antiplasmin
ADP	Adenosine diphosphate
APC	Activated protein C
aPTT	Activated partial thromboplastin time
AT	Antithrombin
ATP	Adenosine triphosphate
AU	Aggregation units
AUC	Area under the curve
Ca^{2+}	Calcium
CBC	Complete blood cell count
C1-INH	C1 inhibitor
et al.	et alii
etc.	et cetera
FV	Factor V
FVa	Activated factor V
FVII	Factor VII
FVIIa	Activated factor VII
FVIII	Factor VIII
FVIIIa	Activated factor VIII

FIX	Factor IX
FIXa	Activated factor IXa
FX	Factor X
FXa	Activated factor X
FXI	Factor XI
FXIa	Activated factor XI
FXII	Factor FXII
FXIIa	Activated factor XII
FXIII	Factor XIII
FXIIIa	Activated factor XIII
FDPs	Fibrinogen or fibrin degradation products
G	Gauge
GPIb-IX	Glycoprotein -Ib-IX-complex
HK	High molecular weight kininogen
i.v.	Intravenous
LRP	Low density lipoprotein receptor-related protein
MEA	Multiple electrode aggregometry
MPC	The mean platelet component
MPM	Mean platelet mass
MPV	Mean platelet volume

PAI-1	Plasminogen activator inhibitor 1
PAI	Plasminogen activator inhibitors
PC	Protein C
PCI	Protein C inhibitor
PK	Prekallikrein
PLT	Platelet
PRP	Platelet-rich plasma
PS	Protein S
PT	Prothrombin time
PZ	Protein Z
rpm	Rotations per minute
sec	Seconds
TEG	Thrombelastography
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
t-PA	Tissue type plasminogen activator
TT	Thrombin Time
TT_{Clauss}	Thrombin time after the Clauss method
u-PA	Urokinase type plasminogen activator
vWf	Von Willebrand factor

LIST OF INCLUDED PUBLICATIONS:

1) „Influence of blood collection technique on platelet function and coagulation variables in dogs", N.Bauer, E.Er, A.Moritz, American Journal of Veterinary Research, Vol.72, No.1, January 2011

2) "Effect of submaximal aerobic exercise on platelet function, platelet activation, and secondary and tertiary hemostasis in dogs", N.Bauer, E.Er, A.Moritz, American Journal of Veterinary Research, accepted October 26,2010

1 INTRODUCTION

1.1 PREFACE

The development of thrombosis and predisposition for thromboembolic diseases are of great interest in human medicine and recently also in veterinary medicine (Bauer N., Moritz A., 2008). Several factors resulting in thrombophilia in healthy individuals including physical exercise have been reported in human medicine (Lippi et al., 2009).

As the knowledge of the impact of sampling on results is important to know for any further investigations, the study was divided in two parts: The aim of the first part ("pre-study") of the study was to investigate the influence of sampling technique on a point of care test (TEG), platelet function, secondary hemostasis (PT, aPTT, fibrinogen, FVIII) as well as physiological anticoagulants (antithrombin, protein C, protein S, APC-ratio) and variables reflecting fibrinolysis (fibrin D-dimers) which has not been reported before in dogs (Part I: Effect of sampling on coagulation variables).

In the second part of the study ("main study"), the influence of standardized submaximal physical exercise on primary hemostasis (platelet activation reflected by ADVIA 2120 platelet activation indices and platelet function assessed by impedance-based aggregometry), secondary hemostasis as well as physiological anticoagulants, variables reflecting fibrinolysis, and kaolin-activated thrombelastography (TEG) parameters determined with re-calcified blood were investigated. Moreover, markers of inflammation (white blood cells=WBC), the hematocrit value and the lactate plasma concentration were of interest (Part II: Effect of submaximal physical exercise on ADVIA 2120™ platelet activation indices, platelet function, secondary and tertiary hemostasis as well as thrombelastography in dogs). The hypothesis was that the coagulation cascade would be activated after submaximal exercise in healthy dogs.

1.2 PHYSIOLOGY OF HEMOSTASIS

1.2.1 Primary Hemostasis

The activation of the coagulation cascade begins with vessel damage resulting in endothelial damage, which leads to the contact between subendothelial matrix, platelets and coagulation factors. Platelet aggregation can generally be divided into five phases: platelet adhesion, platelet aggregation, release of granular contents, facilitation of coagulation and clot reaction (Stockham S.L. and Scott, M.A., 2008). Platelet adhesion to the subendothelial matrix is initiated by a so called “Ligand-Receptor-Interaction”. The Von-Willebrand- factor (vWf) acts as a primary ligand and is mostly synthesized by endothelial cells and megakaryocytes, circulating in the plasma as multimeres of various sizes. The Glycoprotein-Ib-IX-complex on the other side, is the receptor on the platelet surface for this interaction. Under the influence of high flow speed (“shear stress”), the von Willebrand factor (vWf) multimeres unfold, and the interaction vWf-GPIb-IX is induced (Pötzsch et al., 2002).

This is followed by platelet aggregation. Thrombin and other substances such as prostacyclin, thromboxane and adenosine triphosphate (ATP)/adenosine diphosphate (ADP) induce the aggregation of the adhered platelets. At this juncture, the platelets undergo a morphological change (“shape change”). With the contraction of the intracellular cytoskeleton of the platelets, pseudopodes are generated and the intracellular membrane system which draws through the platelets (“open canalicular system”) opens for the platelet surface. Through this system, substances in the organelles of platelets are set free, and the release of granular contents begins. Two organelle types play a major role: α- and δ-granules. α-granules store proteins such as vWF, Factor V (FV), fibrinogen and also other coagulation factors whereas δ-granules contain ATP/ADP, serotonine and histamine (Pötzsch et al., 2002). The stimulation of platelets induces the facilitation of coagulation, and anionic membrane phospholipids which support coagulation move from the inner membrane to the outer membrane, where they are available for use as cofactors in the coagulation pathways. These phospholipids are referred to as “platelet factor 3” and include phosphatidylserine

(Raskin RE, Meyer DJ, 2001, Cowell RL,Tyler RD, 1992). Platelets yield specific binding sites for coagulation enzymes, cofactors and zymogens and also release platelet agonists activating other platelets (Stockham S.L. and Scott, M.A., 2008). Platelets within a thrombus facilitate wound closure and vessel patency by a contractile process involving activated platelets, inter-platelet bridging via the fibrinogen receptor αIIb, β3, and platelet actin and myosin (Cowell RL, Tyler RD, Meinkoth JH, 1999), this is called “clot (coagulum) reaction”.

1.2.2 Secondary Hemostasis

Giving regard to the available coagulation tests, the reactions of the secondary hemostasis can be functionally divided into intrinsic, extrinsic and common pathways (Brooks M, 2010).

1.2.2.1 Intrinsic (Contact) Pathway

The contact pathway consists of the zymogens Factor XII (FXII), also called “Hagemann factor” and prekallikrein (PK), as well as the cofactor high molecular weight kininogen (HK). Activation of the two zymogens occurs spontaneously (auto-activation) upon assembly of the proteins on a negatively charged surface (Colman W., 2006).Contact activation is not calcium dependent and occurs whenever blood is removed from the vascular system. When blood comes in contact with an artificial surface, generation of thrombin and therefore, formation of a fibrin clot is induced and again, contact activation occurs (Brooks M, 2010). Recent work suggests that thrombus formation might have an association with the contact pathway (Gailani D, Renne T, 2007, Muller F, Renne T., 2008). The negatively charged surface which is physiologically relevant and allows contact activation to influence coagulation in vivo has not been detected, although RNA (Kannemeier C, Shibamiya A, Nakazawa F, et al., 2007), polyphosphate (Smith et al., 2006), and misfolded proteins (Maas C, et al. 2008) might be potential agents. The activated platelet surface displays a major role in this pathway and platelet activation is also a component of the function of the intrinsic tenase complex (FIXa-FVIIIa). The factors and their active forms which play a role in the intrinsic

pathway are factor XI (FXI), factor IX (FIX) and factor VIII (FVIII) (Brooks M, 2010). Factor XI is a protein produced in the liver which circulates in the plasma complexed with high molecular weight kininogen (HK) and can be activated by FXIIa, FXIa and thrombin in calcium dependent reactions. Inhibition of FXIa depends on its localization. Plasma inhibitors include its major inhibitor, α_1 protease inhibitor, (α_1-PI), and C1 inhibitor (C1-INH), α_2-antiplasmin (α_2-AP), Antithrombin (AT), protease nexin 1, plasminogen activator inhibitor 1 (PAI-1), and protein C inhibitor (PCI). Platelet bound FIXa is protected from α_1-PI inactivation. Protease nexin 2, together with surface heparan sulfate proteoglycans (HSPGs), plays an important role in the inhibition of FXIa when bound to the endothelial cell surface.This may serve to restrain FXIa mediated cleavage of FIX to the platelet surface (Brooks M, 2010). FIX is also produced in the liver and when calcium is present, tissue factor-FVIIa or FXIa activate FIX bound to a procoagulant membrane surface. The primary substrate for FIXa is FX.The FIXa-FVIIIa complex on a suitable membrane surface (usually the platelet surface) when calcium is present, is a major generator of FXa. The binding of FIX or FIXa to endothelial cells occurs via specific receptors (collagen IV). Binding to the endothelial surface supports activation of FX by FIXa-FVIIIa in the absence of platelets. Factor FIXa alone is able to activate FVII in the absence of TF. FIXa is primarily inhibited by AT-HSPG (Brooks M, 2010).

The initial site of FVIII production is unclear, although the liver and the reticuloendothelial system are strongly implicated. Binding of FVIII to vWf markedly improves the intracellular and plasma stability of FVIII. Thrombin cleavage of FVIII activates FVIII to FVIIIa and releases it from vWf. FVIII can also be activated by FXa, although the maximal activation by thrombin is greater than FXa.Factor VIIIa is cleared from the circulation by low density lipoprotein receptor-related protein (LRP), a common cell surface-receptor found on a variety of cell types.Proteolytic cleavage by activated protein C (APC) and FXa also inactivates FVIIIa (Kaufman et al.,2006).

1.2.2.2 Extrinsic (Tissue factor) Pathway

Tissue factor (TF), also known as thromboplastin, CD142, and factor III, is a glycolysated single polypeptide chain, which is synthesized in a variety of cells, and expressed on the cell surface as an integral membrane protein. In physiologic circumstances, adventitial cells surrounding blood vessels larger than capillaries express TF, differentiating skin keratocytes, and by other epithelial cells, particularly in mucous membranes and organ capsules (Brooks M, 2010). Cytokines and other inflammatory mediators can upregulate TF expression. Factor VII (FVII) is a glycolysated single-chain polypeptide which is vitamin K- dependent and is produced in the liver. Alongside proteolysis, the FVII zymogen is converted to active FVIIa, which can be generated by a variety of downstream proteases, including FIXa, FXa, FVII activating protease, thrombin and plasmin. The most important physiologic FVII activator has yet to be definitely determined (Morissey JH, Mutch NJ, 2006). TF is the regulatory subunit of the TF-FVIIa enzymatic complex, which is the first and most potent activator of coagulation (Weiss&Wardrop, 2010). TF also acts as a co-factor which markedly increases the reaction rate of the FVIIa mediated activation of the proenzymes FX and FIX. Approximately 1% of FVII circulates in the plasma as an enzymatically active form. This differentiates FVII from other coagulation factors which are only available as inactive co-factors. The tissue factor pathway inhibitor (TFPI) constitutes a specific inactivation of FVIIa and FXa (Pötzsch et al., 2002).

1.2.2.3 Common Pathway

Factor X (FX) is activated to FXa by either extrinsic tenase (TF-FVIIa) or intrinsic tenase (FIXa-FVIIIa) on a suitable membrane surface in the presence of calcium, subsequently FXa forms the prothrombinase complex with its cofactor FVa. FXa is inhibited by AT-HSPG and by tissue factor pathway inhibitor (TFPI) as well as by the enzyme/cofactor protein Z-dependent protease inhibitor (ZPI)/protein Z (PZ) (Brooks M, 2010). Factor V (FV) is activated to FVa by thrombin cleavage or FXa, FXa and FVa form an enzyme/cofactor complex referred to as the prothrombinase complex.Proteolysis of FVa by activated protein C (APC) results in its inability to bind prothrombin, which is synthesized in the liver and is the second most

abundant coagulation protein in plasma , after fibrinogen (Jenny NS, Lundblad RL, Mann KG, 2006), and therefore loss of function by the prothrombinase complex.Inactivation of FVa by APC occurs more rapidly on endothelial cells than on activated platelet surfaces (Esmon , 2006, Kane WH, 2006).

Thrombin processes mostly the formation of fibrin by cleaving two short peptides from the fibrinogen molecule, thus revealing binding sites that interact with pre-existing sites on other fibrin molecules, which leads to the polymerization into an insoluble fibrin gel.The polymerized fibrin acts as a cofactor for Factor XIII (FXIII), markedly increasing the rate of FXIIIa generation, whose primary function is crosslinkage of fibrin fibrils (Brooks M, 2010).

Nevertheless, it should be noted that this classification of the secondary hemostasis is more functional and given consideration to the new understanding of hemostasis, a cell based model of hemostasis can be made (Brooks M, 2010).

1.2.3 Tertiary Hemostasis/Fibrinolysis

To avoid and control the spreading of the initial thrombus formed with the reactions of the primary and secondary hemostasis, an inhibition of coagulation is needed. The major coagulation inhibitors include tissue factor pathway inhibitor (TFPI), C1-inhibitor, heparin, antithrombin (AT), protein C, protein S, and thrombomodulin (Brooks M, 2010).

Antithrombin (AT) is produced by the liver, and its endogenous activation occurs when it binds to the heparans on the surface of the endothelial cell. AT neutralizes the activated coagulation factors thrombin and FXa together with heparin. The thrombin-AT complexes are phagocytised by macrophages in the liver.

The protein C- system is a negative feedback mechanism and controls the formation of thrombin which is induced by the binding of thrombin to the co-factor thrombomodulin (TM). Bound to TM, thrombin loses its ability to activate fibrinogen, FV, FVIII, FXI and platelets, on the other hand leads to formation of activated protein C (APC) from the proenzyme protein C. The co-factors FV and FVIII are inactivated by the multienzymecomplex consisting of the APC and the plasmatic co-factor protein S.

Another mechanism of regulation in the coagulation cascade is the fibrinolysis. The tissue type plasminogen activator “t-PA” and urokinase type plasminogen activator “u-PA” enhance the change from the proenzym plasminogen to plasmin and finally, Lys- and Glu-plasmin. After the degradation of cross-linked fibrin, fibrin degradation products such as D-Dimers are set free. The plasminogen activity is blocked by Plasminogen activator inhibitors (PAI) such as α_2–antiplasmin (α_2 –AP) (Brooks M, 2010).

1.2.4 Counter regulation mechanism of primary hemostasis

Prostacyclin and thromboxane are adversely acting prostaglandins synthesized from arachidonic acid. As previously described, thromboxane is a platelet activating agonist. Prostacyclin is as potent as thromboxane and is released from intact endothelial cells, so that the activation of platelets is prevented (Konstantinos Petidis et al., 2008).

1.2.5 Hemostasis during exercise

Human studies have shown that physical exercise in healthy individuals primarily resulted in an activation of the coagulation cascade, resulting subsequently in a counter regulation (Lippi et al, 2009). This exercise induced activation of the coagulatory system (Fehrenbach et al., 2006) is p ossibly caused by an increased immunologic response due to muscular microtraumata which has been observed after strenuous exercise in athletes (Sorichter et al., 1997).

Examinations performed in vivo revealed that macrophages activated with lipopolysaccharides induce an inflammatory reaction, leading to a possible activation of the coagulatory system (Lippi et al., 2004). Furthermore, in marathon runners an increased coagulation activity has been reported starting from 30 minutes until the next morning after the run (Sumann et al., 2007). The changes of coagulation included a shortening of one stage prothrombine time and activated partial coagulation time as well as a significant increase of physiological inhibitors of coagulation (protein S, protein C, antithrombin) and a significant raise in fibrin D-dimer plasma concentration indicative of an increased fibrinolysis (Sumann et al., 2007). Not only maximal exercise such as marathon runs but also prolonged

submaximal physical exercise on a treadmill resulted in an increased evidence of fibrinolysis due to an activation of the coagulatory system (Hilberg et al., 2003) as well as the performance of three maximal short term exercises of 15, 45 and 90 seconds respectively (Hilberg et al., 2003). Besides an activation of secondary hemostasis and fibrinolysis, there was also evidence of exercise-induced platelet activation in healthy athletes as reflected by ADVIA 2120 platelet activation indices (Kratz et al., 2006). Due to sphering of platelets and two dimensional laser light scattering, the hematology analyser ADVIA 120/2120™ with species specific software (Siemens Healthcare Diagnostics GmbH) provides a unique methodology to specifically assess platelets and platelet activation indices. 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 (MPC) and the platelet component distribution width (PCDW) (Ahnadi et al., 2003, Boos et al. 2007, Giacomini et al., 2003). The MPC is a measure for platelet refractive index reflecting platelet granularity – and thus activation status (Moritz et al., 2003). In people and dogs, MPC correlated well with P-selectin (Ruf et al., 1995, Corash et al., 1990, Abrams et al. , 1990). In people, exercise resulted in a decrease of the ADVIA 120/2120 platelet activation indices MPC, MPM and PCDW reflecting an increased platelet activation (Kratz et al., 2006). Comparable studies have not been performed in dogs so far, however, a significantly increased activatability of platelets was reported in sled dogs after strenuous exercise (Moritz et al., 2003).

1.3 Hemostasis and preanalytical factors

Dogs serve as common models for evaluating human diseases, however, a recent study demonstrated that the human coagulation system is closest to sheep, but there are differences between dogs and people (Höhle et al., 2000). Thus, the interaction between physical exercise and coagulation is not necessarily the same in dogs and humans. However, the potential prothrombotic risk of physical exercise is important to know for dogs to estimate the risk of developing thromboembolism and to get an insight into possible

prophylaxis. With the activation of the coagulation process being the interest of the main study, pre-analytical errors due to sample taking should be avoided.

The optimum technique of blood sampling has been widely accepted as atraumatic venipuncture which has been considered to minimize activation of the coagulation cascade and provide the most accurate measurements (Mischke et al., 2000). In people, techniques have been developed to collect blood samples by use of indwelling arterial catheters (Laxson et al., 1994, Templin et al., 1993, Cannon et al., 1985, Heap et al., 1997) and peripheral or central venous catheters (Lindley et al., 1994, Pinto et al., 1994, Powers et al., 1999, Arrants et al., 1999) to avoid unnessessary trauma to a patient´s veins. The influence of sampling technique on coagulation parameters has been rarely investigated in animals. A study in dogs demonstrated that determination of coagulation parameters (PT, aPTT, antithrombin, protein C) and variables indicating inflammation in both venous and arterial blood samples was not associated with a significant impact on the results, however differences in fibrinogen concentration and thrombin time were detected (Paalsgard-Van Lue et al., 2007). Others reported an acceptable aggreement between measurements of prothrombin time (PT), activated partial thromboplastin time (aPTT), and fibrinogen concentration collected via an indwelling i.v. catheter and direct venipuncture (Maeckelbergh et al., 2008, Millis et al., 1995).

However, only a limited number of variables reflecting coagulation activity have been evaluated in these investigations. Furthermore, the diameter of the needle used for sampling was not taken into account as samples have been either collected with a 20 gauge (G) or 22(G) needle. In people, the effect of sampling techniques on coagulation has been investigated in several studies. There was evidence that an increase in shear stress may induce the shedding of procoagulant containing platelet microparticles (Holme et al., 1997, Sakariassen et al., 1998) and therefore contribute to the formation of thrombosis (Miyazaki et al., 1996). The evaluation of shear stress on coagulation is of special interest in people as it commonly caused by severe stenosis of arteries and arterioles by arteriosclerotic plaques (Holme et al., 1997). For this reason, it can be hypothesized that sampling with catheters

with different diameters may result in different degrees of shear stress and - thus an activation of the coagulation system.

The induction of pre-analytical errors, i.e. artificial activitation of coagulation by sample taking is important to know in clinical and experimental settings, however, only rare information is available for dogs.

1.4 Coagulation Tests

1.4.1 Primary Hemostasis

1.4.1.1 ADVIA 120/2120 Activation Indices

The platelet (PLT) cytochemical reaction in the ADVIA 120/2120 system consists of two steps: PLTs are isovolumetrically sphered with dodecyl sulphate so that the sphering eliminates shape as a variable. In the second step, PLTs are fixed with glutaraldehyde and analyzed with a two-dimensional method: the low angle light scatter (2-3°) signal is amplified 30 times, and the high angle light scatter (5-15 °) signal is amplified 12 times. The Mie theory of light scattering for homogeneous spheres is used to convert low- angle light scatter measurement into cell volume and high-angle light scatter into refractive index (Moritz A, Becker M, 2010).

The mean platelet component concentration (MPC) estimates the average platelet density and is used to assess platelet activation status (Chapman ES et al., 1996). The mean platelet volume (MPV) is derived from the platelet volume histogram, whereas the mean platelet mass (MPM) is calculated from the platelet dry mass histogram (= mean of platelet volume x platelet content/100).The platelet component concentration distribution width (PCDW) is a measure of the variability of platelet density, and values decrease with platelet activation (Russell K E, 2010).

1.4.1.2 Whole Blood Aggregometry

Whereas for the majority of coagulation parameters automated assays are available, most platelet function tests cannot be performed automatically. Point-of-care platelet function tests include the platelet function analyser (PFA 100, Siemens Healthcare Diagnostics, formerly Dade Behring) and thrombelastography, e.g. TEG®. The PFA 100 which has been considered in people to be consistent with an in vitro bleeding time (Peters et al., 2001) was used in canine (Keidel et al.,1998, Mischke et al.,2003, Mischke et al., 2002, Tarnow et al., 2003, Callan et al.,2002), equine (Segura et al.,2005), and porcine (Escudero et al.,2001) blood specimen. However, it has the disadvantage that cartridges with standardized agonist concentration have to be used. It is well known that platelet function has to be estimated by using agonists at low to high concentrations (Ozaki et al., 1998). If aggregation response at low concentrations is higher than normal, platelet function is likely to be increased (Ozaki et al.,1998). The TEG has been considered a suitable global coagulation test in dogs to detect an increased coagulation activity (Wiinberg et al., 2008), however, an increased platelet function cannot be distinguished from other reasons of hypercoagulability.

The most commonly used method for platelet function testing is light transmission aggregometry (Born method) employing citrated platelet-rich plasma (PRP). Disadvantages of the Born method include the need of preparation of PRP resulting in a seperation of other blood cells from platelets which are also known to influence platelet function (Santos et al.,1991, Bartlett et al.,1977). Moreover, it has been demonstrated in people that 10%-39% of platelets may be lost during the preparation of PRP, depending on the seperation methods used (Persidsky et al.,1982, Reiss et al., 1976, Hill et al., 1988). Giant platelets which may be both hypo- and hyperactive are generally not included in PRP which may alter the measured platelet-aggregation response in addition (Dyszkiewicz-Korpanty et al., 2005). Therefore, whole blood methods such as impedance aggregometry have been introduced which had the advantage that assessment of platelet function could also be performed in lipemic blood samples. However, impedance aggregometry as performed with older instruments has the draw-back that re-usable electrodes are used which have to be cleaned between several analyses so that they are considered to be impractical and a possible source

of error (Toth et al., 2006). For these reasons, a novel impedance aggregometer (Multiplate® analyser, Verum, Munich, Germany) was developed which was capapable to determine platelet function in diluted whole blood by using disposable test cells with duplicate impedance sensors (Toth et al., 2006). Due to its ease of use, the Multiplate® analyser was considered suitable as point-of-care device in people (Gorlinger et al., 2008) and has been recently evaluated for dogs (Kalbantner et al., 2008). As four electrodes per test cell were used, the method was named multiple electrode aggregometry (MEA) (Toth et al., 2006). Recently, it could be demonstrated in people (Toth et al., 2006) and dogs (Kalbantner et al., 2008) that the use of the thrombin antagonist hirudin as an anticoagulant is preferable to the use of of citrate as it preserves the physiological concentration of ionized calcium and magnesium whereas sodium citrate is known to reduce the calcium concentration in the sample form the millimolar range to micromolar concentrations.

For assessing platelet function, whole blood aggregometry can be performed using an impedance-based platelet function analyzer. Aggregometry is done automatically with single-use test cells consisting of two incorporated sensor units, each with two metal electrodes.

The attachment of platelet aggregates on the electrodes results in an increase of the impedance between them which is recorded as aggregation curve and plotted against the time. The area under the aggregation curve (AUC) is analyzed automatically by the computer attached to the aggregometer by multiplying the maximal amplitude of the curve (AU) with the time (min): AUC=[AU*min] (figure 2). For easier understanding by the clinician, the unit “AU*min” is automatically converted in novel aggregation units (U) according to the formula: 10 AU*min=1 U and reported by the analyser. Maximal increase of the aggregation curve (velocity) is given as AU/min (Figure 1).

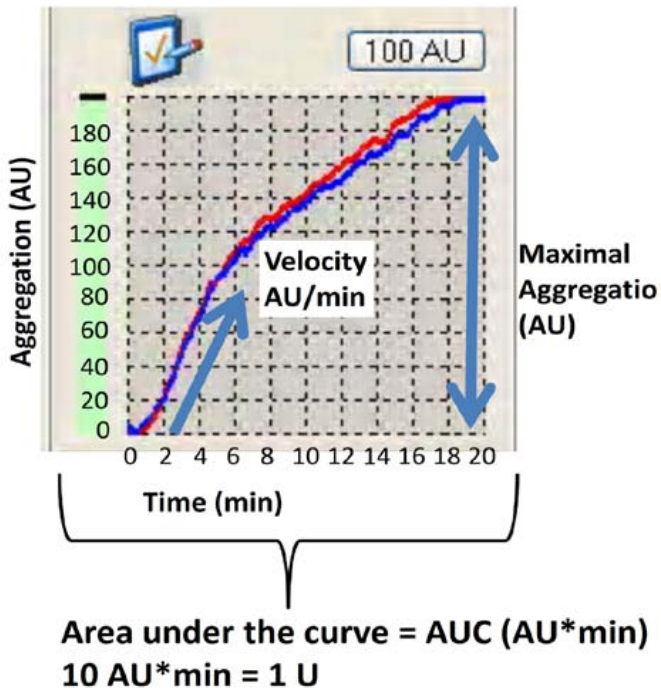

Figure 1 Screenshot of an aggregation curve with the Multiplate® whole blood impedance-based aggregometer.

1.4.2 Secondary Hemostasis

1.4.2.1 Prothrombin time (PT)

The PT is a screening coagulation test of the tissue TF and common pathways where a measured amount of prewarmed Ca^{2+}-thromboplastin reagent is added to a defined amount of prewarmed citrated plasma so that TF will activate factor VII, activation should proceed along the common pathway so as to form fibrin monomers which polymerize to form an insoluble fibrin clot that can be detected optically or electromechanically. The time from plasma-thromboplastin mixing to clot detection is the PT (Stockham, 2008).

1.4.2.2 Activated partial thromboplastin time (aPTT)

The aPTT is a screening coagulation test of the surface-induced and common pathways. Citrated test plasma is incubated (37°C) with an excess of procoagulant phospholipids (partial thromboplastin) and a surface activator (e.g., kaolin, silicates, or ellagic acid) to activate the contact factors. The chelation of Ca^{2+} by citrate in the samples limits activation beyond factor XIa. After a defined incubation time, a defined amount of prewarmed (37°C) calcium chloride is added to counteract the effects of citrate and enable the cascade of coagulation to proceed to the formation of fibrin monomers, these polymerize to form an insoluble fibrin clot that is detected optically or electromechanically. The time from addition of calcium chloride to clot detection is the aPTT (Stockham, 2008).

1.4.3 Tertiary Hemostasis/Fibrinolysis

1.4.3.1 Thrombin time (TT)

The TT asseses the thrombin-induced conversion of fibrinogen to an insoluble fibrin clot without being affected by thrombin generation. Thrombin is added to prewarmed test plasma (37°C).Thrombin cleaves fibrinogen to form monomers, which polymerize into an insoluble fibrin clot that can be detected optically or electromechanically. The time from thrombin addition to detection of a fibrin clot is the TT (Stockham, 2008). The Clauss method (TT_{Clauss}) uses diluted plasma and high thrombin concentrations to better measure functional fibrinogen with less interference by heparin and fibrin or fibrin degradation products (FDPs) (Bateman et al., 1999).

1.4.3.2 Fibrinogen

Fibrinogen is calculated from TT_{Claus}, where TT_{Claus} values of samples with a known fibrinogen and therefore activity are used to construct a reference standard curve from which a test plasma fibrinogen can be estimated. TT_{Claus} is measured in seconds, but values for fibrinogen are read from the reference standard curve and reported in mg/dL or µmol/L (Stockham, 2008).

1.4.3.3 Antithrombin (AT or ATIII)

Plasma AT is usually measured by chromogenic (functional) assays rather than immunoassays that detect AT antigen but not function (Bateman et al., 1999). Test plasma is added to a reagent containing heparin, excess thrombin or factor Xa (depending on the assay), and the corresponding chromogen-labeled substrate for thrombin or factor Xa. Increased AT in the test plasma causes less activity of thrombin or factor Xa and therefore less color change (measured spectrophotometrically) (Stockham, 2008). Units are % activity compared to either a species-specific or human plasma pool considered to have 100% activity. Because of species differences in AT activity, reference an reported values vary with the species used for the reference pool (Pusterla N et al., 1997, Mischke R et al., 2000, Green RA, 1988).

1.4.3.4 Fibrin(ogen) degradation products (FDPs)

To measure D-dimers, immunoturbidimetric tests such as the Liatest D-Di test (Roche, Mannheim) can be used which has been validated previously for its use in dogs (Bauer et al.,2009). In this assay, a microlatex suspension (chloro-methyl-polystyrene-latex particles of 0.1 ± 0.02 µm) is coated covalently with two complementary monoclonal antibodies specific for fibrin D-Dimer epitopes within X-oligomeres (Oger et al., 1998). The assay is performed fully automated by mixing 50 µl of undiluted citrated plasma with 100 µl of reaction buffer for 4 min at 37° C and the test is initiated with 150 µl of latex suspension. Agglutination caused greater light scattering which was detected as an increase in optical density. The change in absorbance, measured at 540 nm is automatically recorded for 140 s and represented a direct relationship to the fibrin D-dimer concentration in the specimen. The D-dimer result is reported as mg/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 is pre-calibrated and allowed a one-time testing on an automated benchtop analyzer (Oger et al, 1998).

1.4.3.5 Protein C/Protein S

Protein C and Protein S has been infrequently assayed in veterinary medicine but provides information about a patient's anticoagulant and fibrinolytic status. Low plasma concentrations or activities predispose individuals to thrombosis because of decreased inactivation of factors Va and VIIIa and because of decreased fibrinolysis. Activity can be measured with the use of canine pooled plasma for calibration by clot-based functional assays (Welles EG et al., 1991) or colorimetric (chromogenic substrate based) assays (Fry, 2011).

1.4.3.6 APC Ratio

The principle of assessment of APC resistance is based on an unusually small prolongation of the clotting time of the tested plasma in the presence of activated protein C and in calcified medium. Patient plasma is diluted and coagulation is achieved in the presence of factor V deficient plasma and *Crotalus viridis helleri* venom which acted as an activator of factor X. Thus, it triggers the coagulation cascade downstream from factor X so that an influence of all coagulation factors acting upstreams could be ruled out. The prolongation of the clotting time of normal plasma in the presence of activated protein C resulted from the capacity of activated protein C derived from the test kit to inactivate the factor Va of the tested sample (Svensson et al, 1997). APC resistance is expressed in the form of a ratio, reference intervals for dogs have been described previously (Bauer et al., 2009).

1.4.4 Thrombelastography (TEG) as a global test

The TEG allows an assessment of global hemostatic function with whole blood by evaluation of both interaction of cellular and plasma components during initiation, amplification and propagation of clot formation as well as during fibrinolysis. The advantages of TEG include the assessment of overall hemostatic function by evaluation of the visco-elastic properties of the developing blood clot, including clot formation, kinetics, strength, stability and

resolution as well as immediate and cage side detection of hyper- and also hypocoagulable states (Wiinberg et al., 2008).

Interpretation of TEG results:

Five TEG variables (R, K, angle α, MA and G) are evaluated which have been published before (Wiinberg et al., 2008, Wiinberg et al., 2007, Wiinberg et al. 2005).

R is the reaction time and represents the time of latency from the time the blood was placed in the TEG analyzer until a present fibrin formation is reached, measured as an increase in amplitude of 2 mm. *R* is primarily related to plasma clotting factors and inhibitor activity. The clotting time *K* is the time to clot formation measured from the end of *R* until an amplitude of 20 mm is reached. It is a measure of the time it takes from initial clot formation until a predetermined clot strength is reached. *K* is primarily related to clotting factors, fibrinogen, and platelets. The *α* angle represents the rapidity of fibrin build-up and cross-linking and is mainly dependent on platelets, fibrinogen concentration, and clotting factors (Wiinberg et al., 2005).

MA is the maximum amplitude reached. The MA is dependent on platelet count and function as well as fibrinogen concentration, and it is a direct function of fibrin and platelet bonding, which represents the ultimate strength of the fibrin clot. The TEG maximum amplitude also is a measure of clot stiffness and can be used to derive clot shear elastic modulus G, where G= 5.000 x MA/(96 - MA) and is a measure of the overall coagulant state as normo-, hyper-, or hypocoagulant (Figure 2) (Wiinberg et al., 2005).

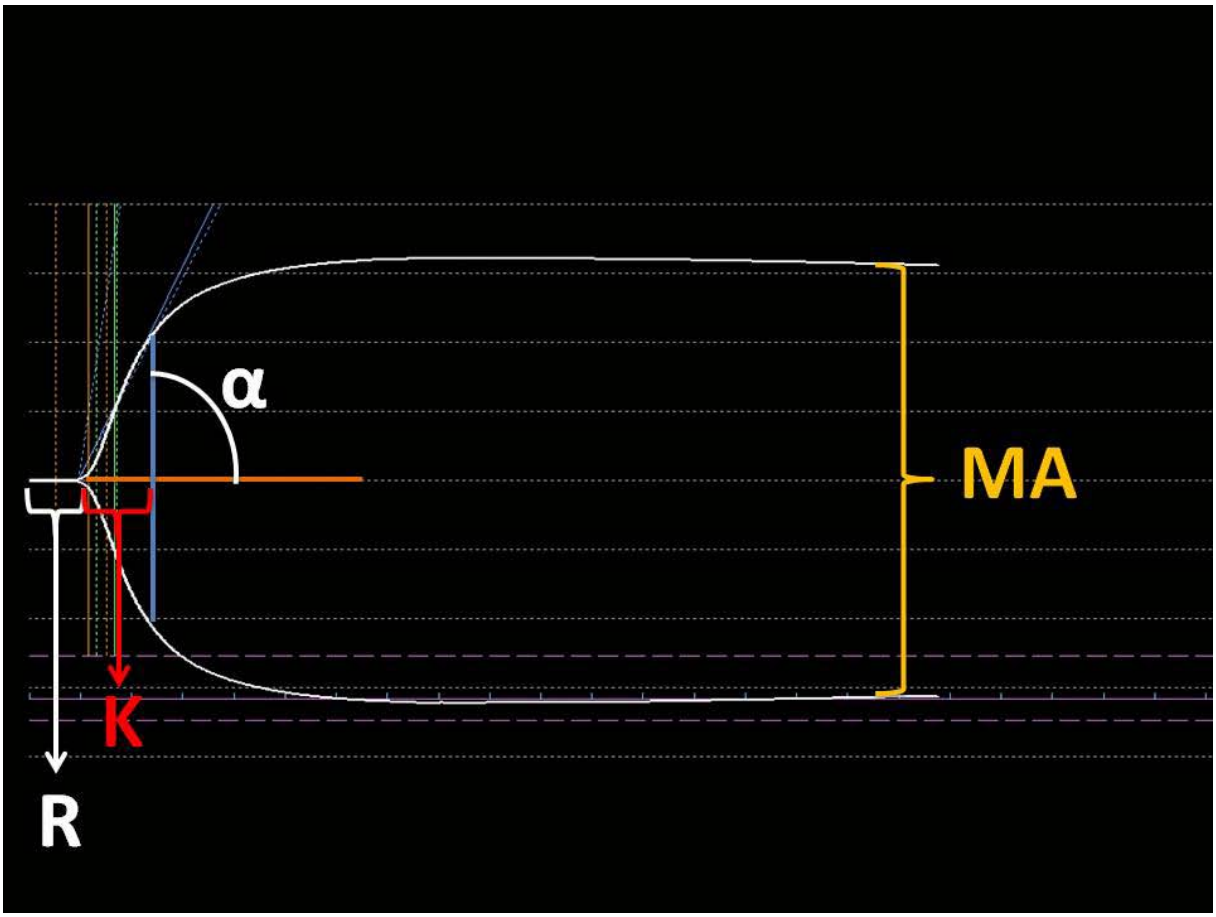

Figure 2 : TEG curve and parameters.

2 MATERIAL AND METHODS

2.1 Part 1: Effect of sampling on coagulation variables

2.1.1 Study Design

The prospective investigation was ethically approved by the Ethics Committee for animal welfare, Giessen, Germany (No. V54-19c20-15 (1) GI18/17-No.30/2006).

Platelet function was assessed with the impedance-based whole blood platelet function analyzer Multiplate®[1]. Variables characterizing secondary hemostasis, physiological anticoagulants and markers of fibrinolysis were obtained with the automated coagulation analyser STA Compact®[2]. Assays run on the STA Compact included a standard coagulation profile consisting of coagulation times (PT, aPTT), 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). A kaolin-activated TEG analysis[3] was performed in addition.

2.1.2 Dogs

The study was performed in 6 clinically healthy Beagle dogs (4 male neutered, 2 female neutered) with a median age of 2.5 years (range 2 - 3 years).

In each dog, a physical examination was performed as well as a complete haematological and clinical chemical examination 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 concentrations.

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[1] Multiplate ®,Verum, Munich, Germany

[2] STA Compact®, Roche Diagnostics GmbH, Darmstadt, Germany

[3] TEG® 5000 Thrombelastograph, Haemonetics Corporation (formerly Haemoscope Corporation), Braintree, MA, USA

2.1.3 Sampling

Samples were obtained simultaneously from fasted, resting dogs with a 20G intradermic needle[4] (technique 1) at one cephalic vein, a 18 gauge (G) x 45 mm venous catheter[5] (technique 2) placed in the other cephalic vein, and two central venous indwelling catheters including one 14G x 16 cm radiopaque polyurethane catheter inserted with Seldinger technique[6] (technique 3) and a 13G catheter[7] (technique 4) placed with the "over-the-needle" method. In this study, each dog served as its own control. Sampling was always performed in the same order, i.e. first with the venous catheter, then the needle, the central venous catheter placed with Seldinger technique and last the central venous catheter inserted with the over-the-needle technique. Samples were taken every 10-15 minutes (maximal 30 minutes).

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. The first 2 ml were discarded to remove tissue thromboplastin and used for hematological and clinical chemical analysis.

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 carefully mixed.

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

[4] Neoject 20-gauge Dispomed Witt oHG,Gelnhausen,Germany

[5] Vasoflo-int, intravenous opaque catheter with injection valve, Dispomed Witt oHG,Gelnhausen,Germany

[6] Central venous catheterization set with Blue Flextip catheter, 14-gauge, 16-cm catheter length,0.032-inch diameter spring wire guide,Arrow, International Inc., Pa

[7] Vygoflex Pur®, 1.2x1.7 mm / 35 cm, article number 9152.517, Vygon GmbH&Co KG, Aachen, Germany

and stored at -80°C until analysis. Analysis was performed within 3 weeks after sampling. Sample stability was proven 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.

For aggregometry with the Multiplate® platelet function analyser, venous blood was placed in a 4.5 ml blood collection tube containing the thrombin inhibitor hirudin in a concentration of 25µg/ml[8]which was obtained from the manufacturer. Platelets were allowed to rest 30 minutes prior to analysis.

2.1.4 Test methods applied at the STA Compact

Directly before the analysis, plasma samples were thawed at 37°C in a water bath and centrifuged at 850g for 10 minutes to remove remnants of cryoprecipitate after thawing.

Coagulation screening tests PT and aPTT were measured automatically as clotting tests using commercial reagents[9]. Results were reported in seconds. Fibrinogen was detected with the Clauss method[10]using a human plasma calibration standard[11]provided by the manufacturer.

Fibrin D-dimers were measured with the STA Liatest D-Di™ immunoturbidimetric D-dimer assay[12]. Antithrombin activity was detected based on its inhibition of thrombin with a chromogenic substrate kit[13]. AT activity was reported as percentage of human plasma calibration standard[14]. For measurement of protein C, an automated clotting test was performed[15]: Cephalin, a phospholipid PF3 equivalent (50 µl), human protein C deficient plasma (50 µl) and 50 µl of a specific protein C activator (Protac) were added to 50 µl the

[8] Thrombin inhibitor blood collection tube 4,5 ml, Verum Diagnostica GmbH, Munich,Germany

[9] STA APTT kaolin, Roche Diagnostics GmbH,Mannheim,Germany

[10] STA fibrinogen, Roche Diagnostics GmBH,Mannheim,Germany

[11] STA Unicalibrator,Roche Diagnostics GmbH,Mannheim,Germany

[12] STA Liatest™ D-Dimer, Roche Diagnostics GmbH, Mannheim, Germany

[13] STA Antithrombin III, Roche Diagnostics GmbH, Mannheim, Germany

[14]STA antithrombin III,Roche Diagnostics GmbH, Mannheim, Germany

[15]STA protein C clotting, Roche Diagnostics GmbH,Mannheim,Germany

patient sample prediluted 1:5 with diluent buffer[16]. This resulted in an activation of protein C within the sample and simultaneously an initiation of the intrinsic clotting system by contact activation. Based on this method, the aPTT was determined solely by the protein C activity in the sample as addition of protein C-deficient human plasma. Activated protein C cleaves factors Va and VIIIa resulting in an increase in aPTT. Thus, protein C activity was directly proportional to the increase in aPTT in seconds. Results were reported as percentage of canine pool plasma calibration standard.

Protein S activity was also determined with an automated clotting test[17]. Fifty µl human protein S deficient plasma, 50µl human activated protein C, and 50 µl bovine factor Va were added to 50 µl of the patient sample which was automatically diluted 1:5 with diluent buffer. As protein S is a cofactor of protein C, the anticoagulatory effect of protein C was solely increased by the protein S activity in the patient sample.

For the measurement of APC Ratio, in the test system provided by the manufacturer, 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 which acted as an activator of factor X. The result - the aPTT in the presence of APC - was recorded in seconds and devided by the aPTT in absence of APC to obtain the APC ratio.

Measurement of FVIII was performed with a modified 1-stage aPTT using a human FVIII-deficient substrate plasma[18]. Routine dilution of patient samples was 1:40 with diluents buffer. In case of FVIII activity > 150%, measurement was automatically repeated in a dilution of 1:60. Results were reported as percentage in comparison to a canine plasma pool.

For all variables except protein C, protein S and factor VIII activity internal quality control material (normal and abnormal) provided by the manufacturer was run each time of measurement. STA PreciClot Plus I and II [19] was used for quality assurance of the majority of variables including PT, aPTT, TT, fibrinogen and AT. A third level (STA PreciClotPlus III, also

[16] STA diluent buffer,Roche Diagnostics GmbH,Mannheim,Germany

[17] STA protein S clotting, Roche Diagnostics GmbH,Mannheim,Germany

[18] STA factor VIII,Roche Diagnostics GmbH,Mannheim,Germany

[19] STA Preciclot Plus I and II,Roche Diagnostics GmbH,Mannheim,Germany

abnormal) was run in addition to PT, aPTT, fibrinogen, and AT. For internal quality control of fibrin D-dimer measurements, Liquicheck™ D-dimer control Level I and II[20]was used. In case of APC response, material for internal quality control was included in each reagent package.

2.1.5 Preparation of canine pool plasma

Protein C, protein S, and Factor VIII activities were determined in comparison to a standard curve derived from dilutions of canine pool plasma. Approximately 30 ml citrated whole blood was taken from 16 healthy adult dogs (8 female, 6 male, 2 female castrated) 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 each were included as well as one Rottweiler, Staffordshire Bullterrier and mixed breed dog each. The dogs were healthy based on the history, physical examination as well as a hematological and clinical chemical examination.

2.1.6 TEG Analysis

The TEG using a TEG5000 analyser[21] 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[22]. Mixing was ensured by gentle inversion of the kaolin-containing vials for 5 times. Pins and cups[23]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 calcium chloride 0.2molar and 340µl kaolin-activated citrated whole blood was added so that a total volume of 360 µl was reached in each cup.

[20] Liquicheck D-dimer control level I and II,Roche Diagnostics GmbH,Mannheim,Germany

[21] TEG 5000 thrombelastograph, Haemonetics Corp.,Braintree,Mass.

[22] TEG® Hemostasis System Kaolin, Haemonetics Corporation (formerly Haemoscope Corporation), Braintree, MA, USA
[23] TEG® Hemostasis System Pins and Cups, Haemonetics Corporation (formerly Haemoscope Corporation), Braintree, MA, USA

Internal quality control materials in two levels (normal and abnormal)[24] were run each day of analysis. An electrical internal quality control (so called e-test) was performed in addition.

2.1.7 Whole blood aggregometry

For assessing platelet function, whole blood aggregometry was performed using an impedance-based Multiplate® platelet function analyzer. Aggregometry was performed automatically with single-use test cells[25] with two incorporated sensor units, each with two metal electrodes. The aggregometer was pre-warmed at 37°C and the test cells were preloaded with 300 µl of 0.9% saline. Then, 300 µl of hirudin-anticoagulated whole blood was added. After an incubation of 3 minutes duration, the agonist was pipetted in the test cell. For induction of platelet aggregation, collagen[26] was used at five different final concentrations, i.e. 0.8 µg/ml; 0.4 µg/ml; 0.2 µg/ml; 0.1 µg/ml; and 0.05 µg/ml. After addition of the agonist, blood was stirred with an electromagnetic stirrer by 800 rpm. After incubation for 3 minutes, 20 µl of the agonist were added to the sample. Activated platelet function was recorded for 20 minutes. Measurements were always performed in duplicates. The mean was calculated automatically by the computer software and was used for statistical analysis.

An electrical internal quality control (so called electronic control) was performed once a day.

[24] TEG® Coagulation Control Level I and II, Haemonetics Corporation (formerly Haemoscope Corporation), Braintree, MA, USA

[25] Single-use test cell, Dynabyte GmbH, Munich, Germany

[26] COLtest, Dynabite GmbH, Munich Germany

2.1.8 Statistical analysis

Results were analyzed with the Graph Pad Prism[27]and Medcalc statistical software[28]. A Kolmogorov Smirnov test was performed to verify the assumption of normality.

The differences between results obtained with the 4 sampling techniques were assessed with a one-way ANOVA test and Levene´s test of variances. Data obtained from whole blood aggregometry were analysed using a two-way ANOVA test after logarithmic transformation of data. Statistical analysis included the effect of sampling and collagen dosage as well as the interaction between both factors.

[27] Graph Pad Software, San Diego, USA

[28] Medcalc® version 9.0.1.1 for Windows - © 1993-2006 Frank Schoonjans

2.2 Part 2: Effect of submaximal physical exercise on ADVIA 2120™ platelet activation indices, platelet function, secondary and tertiary hemostasis as well as thrombelastography

2.2.1 Study Design

The prospective study was approved by the Ethics Committee for animal welfare, regional board Giessen, Germany (V54-19c20-15(1)Gi 18/17 No 91/2009).

The same measurements were performed in each of the 9 dogs to achieve an intra- and inter-individual comparison of the impact of standardized submaximal exercise on the coagulatory system. Each dog in this study served as its own control.

As an indicator of submaximal exercise, the heart rate of the dogs was recorded continuously and plasma lactate levels were measured prior, during and after exercise.

As in the first part of the study, platelet function was assessed with the Multiplate ®impedance-based whole blood platelet function analyzer[29]. Variables characterizing secondary hemostasis, physiological anticoagulant agents and markers of fibrinolysis were obtained with with the STA Compact automated coagulation analyzer[30]. Assays ran on this analyzer included OSPT, aPTT, plasma concentration of fibrinogen, FVIII activity, Antithrombin III, protein C, protein S, and D-dimer plasma concentration. In addition, a kaolin-activated TEG analysis was performed.

2.2.2 Dogs

The study was performed with 9 healthy beagles (5 neutered males and 4 spayed females) with a median age of 4 years (range 2-4 years) and a median body weight of 14 kg (range 10.5 – 18.2 kg) on the time period of study.

The dogs were healthy based on physical examination, complete blood cell count (CBC), blood chemical profile (urea, creatinine, bilirubin, alkaline phosphatase, alanine aminotransferase, glutamate dehydrogenase, sodium, potassium, chloride, ionized calcium,

[29] Multiplate ®, Dynabyte, Munich, Germany

[30] STA Compact ®, Roche Diagnostics GmbH, Darmstadt, Germany

magnesium, phosphate, total protein, albumin, globuline, fructosamine and glucose concentrations). Also, radiographies of the thorax, electrocardiogram (ECG), echocardiography and blood pressure measurements revealed no abnormalities. 4 weeks prior to the study, the dogs were conditioned to run on a treadmill 2 times a week. It should be noted that the dogs were not trained to achieve a specific fitness level.

2.2.3 Submaximal Exercise

Thirty minutes prior to exercise, a 13G indwelling polyurethane catheter[31] was placed in the jugular vein with the "catheter-through-the-needle" technique to avoid excitement due to catheterization during the study period. In all dogs, exercise was performed at ambient temperature (22°C). The exercising of the dogs and the measurements took place at a time span between 08:00 am and 14:00 pm. While the dogs were performing the exercise, they wore a harness and a leash, so that they were running steadily on the treadmill.

The dogs were fasted 12 hours prior to exercise. Thirty minutes after catheterization, the fasted dogs ran on a treadmill[32](See figure 3) at a velocity of 6 km/h. After 3 minutes, the treadmill's slope was elevated by 4° every 2 minutes up to 20° (See Table 1). Blood samples were taken before and immediately after the 13-minute exercise period as well as 60 minutes after ending of the exercise programme. Heart rate was recorded continuously by telemetry[33] (See figure 4). The mean heart rate at each of the three time points was calculated from the last 20 consecutive measurements prior to sampling.

[31] Vygoflex Pur®, 1.2x1.7 mm / 35 cm, article number 9152.517, Vygon GmbH&Co KG, Aachen, Germany

[32] Quasar, H.P. Sportgeräte GmbH, Traunstein, Germany

[33] Polar Sport tester RS400sd, Polar Electro Oy, Professorintie 5, Kempele, Finland

Figure 3: Beagle „Sky“ running on treadmill (slope : 20°) with the attached electrode around the thoracic wall for the continous documentation of the heart rate.

Type of training	Slope (°)	Time (min.)
Rest	0	2
Warming up	0	3
Running	4	2
Running	8	2
Running	12	2
Running	16	2
Running	20	2
Rest	0	60

Table 1: Programme of submaximal exercise

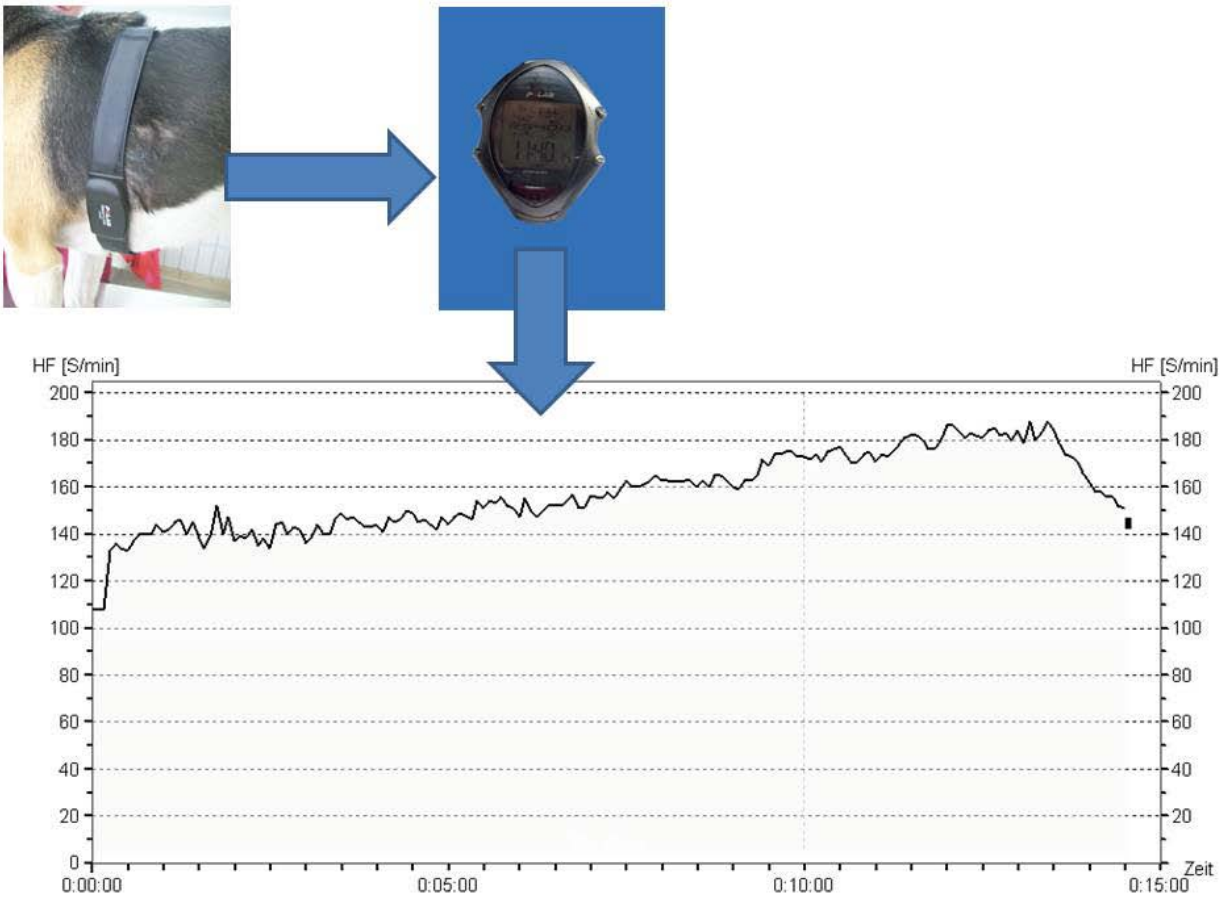

Figure 4: Devices for the telemetry method and and pulse curve during the submaximal exercise programme.

2.2.4 Sampling

The first 4-5 ml of aspirated blood from the central venous catheter was discarded. Then, blood samples were carefully aspirated in 5 ml syringes[34] and gently placed into the anticoagulant containing tubes. After sampling, the central venous catheter was flushed with 0.9 % saline. Blood samples were collected in a 1.2 ml EDTA tube, a 10 ml silicone-lined vacutainer tube containing sodium citrate 3.13 %, a 1.2 ml tube containing 3.18 % sodium citrate, and a 4.5 ml blood collection tube containing the thrombin inhibitor hirudin in a concentration of 25 µg/ml[35]. The EDTA blood sample was used for haematological analysis including the assessment of ADVIA 2120 platelet activation indices. The citrate tubes were inverted carefully several times to ensure mixing citrate and blood in a 1:9 ratio. The samples were carefully checked for proper filling and only specimens with an exact ratio of 1:9 citrate to blood anticoagulant were included. The 1.2 ml citrated tube was stored at room temperature for 60 minutes for following TEG analysis. Sodium-citrated whole blood obtained in the 10 ml Vacutainer tube served for characterization of secondary and tertiary hemostasis and was spun down at 850g for 10 minutes within 1 hour after sampling. Citrated plasma was separated from the erythrocytes and centrifugated again at 850g for 10 minutes to remove all non-sedimented platelets prior to freezing as previously recommended. The supernatant was removed and stored at -80°C until analysis. Analysis was performed within 16 days after sampling. For all analytes, sample stability was proven to be > 12 months. (Bauer et al., 2009). Directly prior to analysis, plasma samples were thawed at 37°C in a water bath as recommended (Bruhn et al., 2007) to provide complete dissolving of cryoprecipitate. Afterwards, the specimens were centrifugated at 850g for 10 minutes so that the plasma was mixed.

Hirudin – anticoagulated blood was used for whole blood aggregometry and platelets were allowed to rest 30 minutes at room temperature prior to analysis.

[34] Ecoject®, 5ml syringe with a polypropylene cylinder and a polyethylene piston, Dispomed, Gelnhausen, Germany

[35] Thrombin inhibitor blood collection tube 4.5 ml, Dynabyte GmbH, Munich, Germany

2.2.5 Lactate plasma concentration

In order to assess the lactate plasma concentration blood was collected in a 1.2 ml tube containing NaF (sodium fluoride). Within 15 minutes after sampling, the samples were centrifugated at 15.000g for 2 minutes and analysis was performed directly after that using a Pentra 400 analyzer.[36]

2.2.6 Test methods for the STA compact

The parameters measured on the STA compact were PT (OSPT), aPTT, antithrombin (ATIII), protein C, protein S, fibrinogen, d-dimer, factor VIII and the APC ratio. The methods used were identical to the methods described in part 1.

2.2.7 TEG Analysis

The method and interpretation of samples were identical as described in part 1.

2.2.8 ADVIA 2120 analysis

The ADVIA 2120 system (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany) was run with the veterinary software version 5.3.1.-MS.

ADVIA 2120 analysis included a measurement of white blood cell count (WBC), hematocrit value, platelet count, as well as platelet activation indices. ADVIA 2120 platelet parameters were measured flow cytometrically in the ADVIA 2120 platelet channel.

Platelet analysis was performed by the measurement of the intensity of two dimensional laser light scattering. Results were then converted to approximate measures of platelet volume and refractive index respectively. Based on these measurements, a variety of platelet activation indices were obtained including the MPV (mean platelet volume, derived from the platelet volume histogram), MPC (mean platelet component concentration, a measurement of platelet density calculated directly from the refractive index), MPM (mean platelet mass, calculated from the platelet dry mass histogram = mean of platelet volume x

[36] Pentra 400, Horiba ABX, Stuttgart, Germany

platelet content/100) and the platelet PCDW (platelet component distribution width, a measure of the variation in platelet shape change, MPC x 100/standard deviation of MPC).

2.2.9 Whole blood aggregometry

Whole blood aggregometry was performed on the same analyzer with the same method used in part 1 only with different concentrations of collagen. For induction of platelet aggregation collagen was used at four different final concentrations, i.e. 10 µg/ml; 5 µg/ml; 3.2 µg/ml; 1.6 µg/ml. Aggregation was also evaluated without any agonist to assess spontaneous aggregation.

2.2.10 Statistical analysis

Results were depicted as Box- and Whisker plots and comparison with laboratory reference intervals was performed if available. For TEG parameters and variables reflecting secondary and tertiary hemostasis, laboratory-intern 95% reference intervals were obtained from 56 healthy adult dogs as described previously (Bauer et al., 2009). For establishment of reference intervals, blood samples were drawn though an 18 gauge (G) venous catheter, however, it was demonstrated previously that there was no significant difference between TEG parameters, results of the impedance based Multiplate analyzer and variables reflecting secondary and tertiary hemostasis when obtained with an 18 G catheter or an 13G central venous catheter inserted with the “through-the-needle-technique” (Bauer et al., 2009). For ADVIA 120/2120 platelet activation indices, reference intervals were established previously (Moritz et al., 2000; Bauer et al.,2009).

The impact of exercise on heart rate, TEG parameters and variables reflecting secondary and tertiary hemostasis was assessed with a one way analysis of variance and covariance with repeated measures. In case of non-normal distribution (large PLTs), logarithmic transformation of data was performed. Due to one missing value at time point 60 minutes, the differences between results obtained at the 3 time points of sampling were assessed with a non parametric Wald test using commercial statistical software for ADVIA 2120 analyses, lactate and the APC-ratio. The exercise-induced changes of whole blood

aggregometry were assessed with an analysis of variance and covariance after logarithmic transformation of data. Co-variables were the collagen dosage as well as the interaction between exercise (i.e. the time point of sampling) and collagen dosage. All analyses were performed with commercially available statistical software[37]. Level of significance was set at $\alpha = 0.0019$ after Bonferroni correction for all statistical analyses.

[37] BMDP Statistical software Inc., 1440 sepulveda Blvd, Los Angeles, CA 90025 USA

3 RESULTS

3.1 Part 1: Effect of sampling on coagulation variables

Generally, the sampling technique did not have a significant impact on all coagulation variables assessed here.

Regarding the platelet function assessed with the Multiplate®, a tendency towards increased platelet activation at low agonist concentrations of 0.05 µg collagen/ml, i.e. higher median AUC was evident in specimens drawn through venous catheters inserted with the Seldinger technique (figure 5, sampling technique 3), however, the difference between the methods was not statistically significant. Median AUC for technique 3 was 46.7U (range 11.3-109.1U), followed by technique 2 (median 26.25U; range 9.0-102.0U), technique 4 (median 18.1U; range 6.4-97.5U) and technique 1 (median 13.3U; range 9.0-99.8U).

At the lowest collagen concentration of 0.05 µg/ml, the highest median velocity was observed for sampling techniques 2 and 3 (4.3 AU/min; range 2.5-6.5 AU/min and 3.7 AU/min; range 2.8-8.3 AU/min, respectively) which, however, was not significantly higher after Bonferroni correction than the results obtained for technique 4 (median velocity 2.7 AU/min; range 1.9-5.1 AU/min; all $p<0.05$).

As expected, the two way test of variance revealed also a significant influence of the agonist concentration on the aggregation AUC and velocity of aggregation. A high overall inter-individual variation of platelet function could be observed as evident in figure 5.

As shown in figure 6, a tendency towards a higher TEG-G value was observed in samples collected through catheters inserted with the Seldinger technique, with the results not being statistically significant. In specimens taken with a 18G venous catheter (sampling technique 2) and a central venous catheter inserted with the over-the-needle method (sampling technique 4), a higher variation of the several TEG parameters including the angle α as well as the TEG-R and K-value was observed in comparison to the other methods of blood collection (figures 7 to 9).

The sampling technique did not have any significant impact on routine coagulation parameters reflecting either secondary hemostasis, natural anticoagulants, or fibrinolysis (figures 10 to 17).

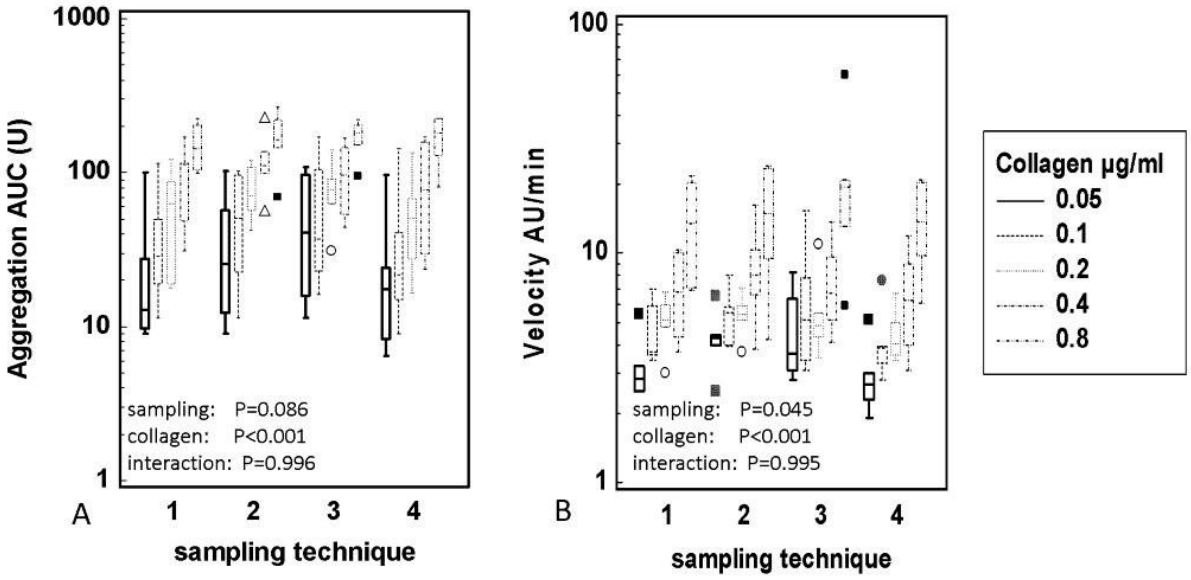

Figure 5: *The effect of the sampling method on platelet aggregation assessed using the whole blood aggregometer Multiplate® (n=6 dogs). Collagen was used as an agonist at dosages ranging from 0.05 to 0.8 µg/ml. Sampling techniques were as follows: 1=20G needle; 2=18G venous catheter; 3=14G venous catheter applied with Seldinger method; 4=13G central venous catheter placed with over-the-needle technique. Analyser-specific variables, i.e., the area under the aggregation curve (AUC) given in novel aggregation units (U) and the velocity of aggregation AU/minute (min) are shown. The values from the lower to upper quartile are presented in the central box. The middle line is consistent with the median. The horizontal line extends from the minimum to the maximum value, excluding "outside" and "far out side" values which are displayed as separate points. Outside values are defined as values that are smaller than the lower quartile minus 1.5 times the interquartile range, or larger than the upper quartile plus 1.5 times the interquartile range. These results are plotted with a square marker. "Far outside" results are consistent with the upper and lower quartile*

*plus/minus 3.0 times the interquartile range.*Abbreviations: AU=aggregation units (maximal amplitude); AUC=Area under the curve; min=minute; U=novel aggregation unit

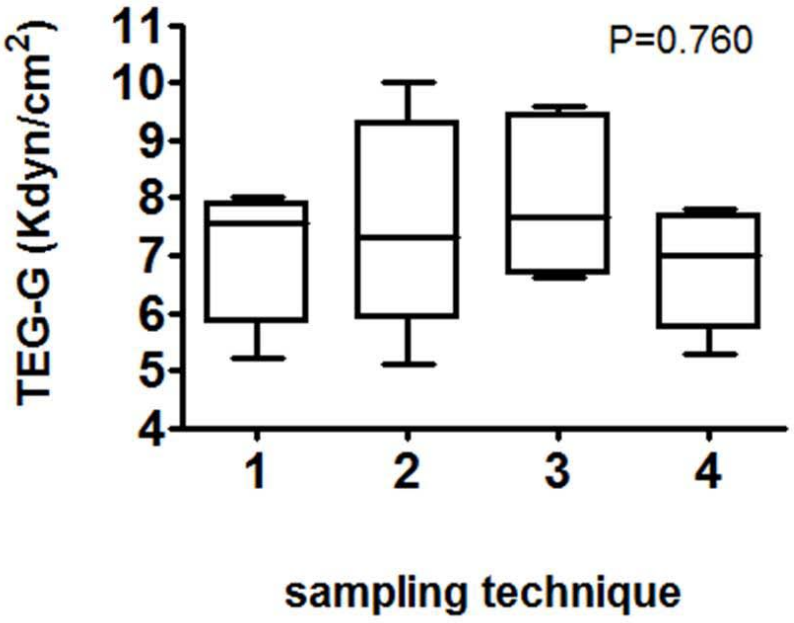

Figure 6 : Box and whisker diagram showing measured G-values in the TEG, for remainder key, refer to figure 5

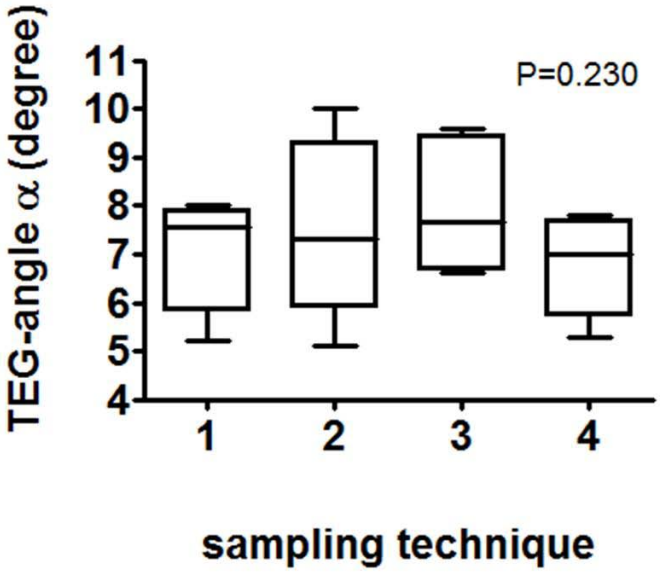

Figure 7: The TEG-α parameter measured with 4 sampling techniques (for remainder key refer to figure 5)

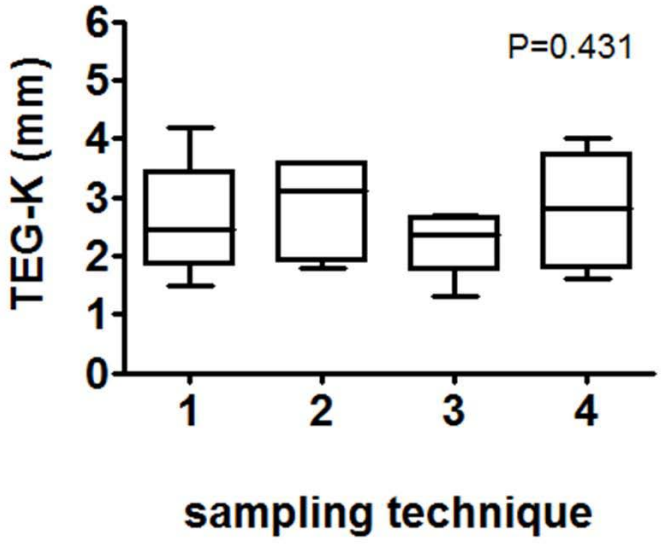

Figure 8:TEG K-value obtained with 4 sampling methods (for remainder key, refer to figure 5)

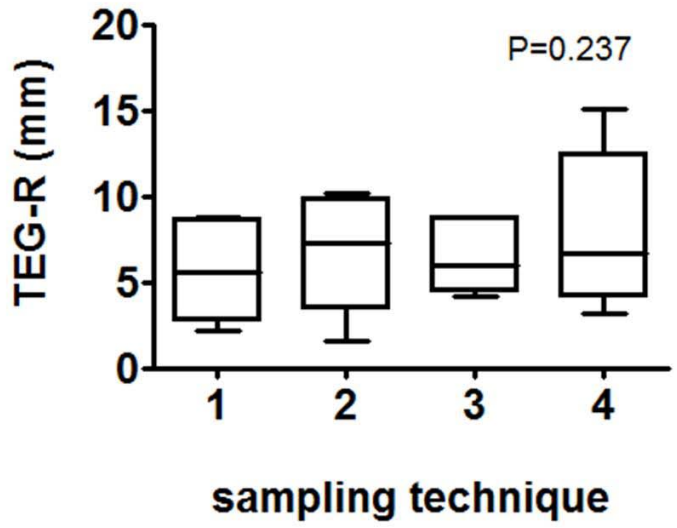

Figure 9: TEG-R value showing no statistical difference between sampling methods (for remainder key, refer to figure 5)

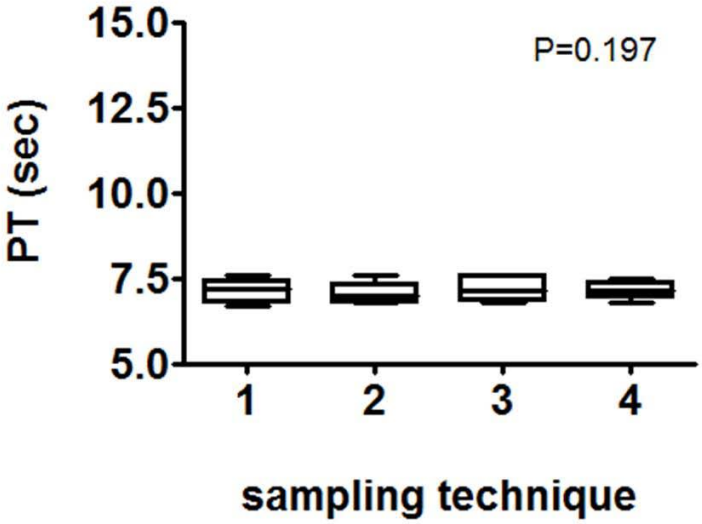

Figure 10: Box and whisker diagram of PT showing no statistical differences regarding the techniques of sampling (for remainder key, refer to figure 5)

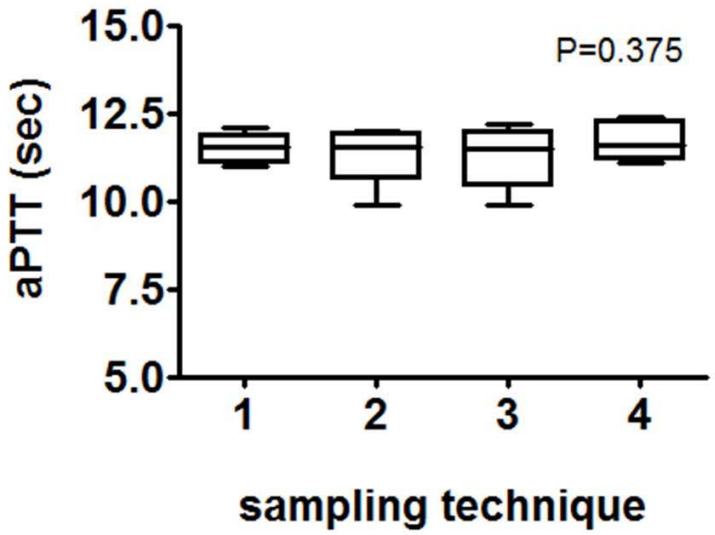

Figure 11: aPTT results showing nosignificant impact of the sampling technique (for remainder key, refer to figure 5)

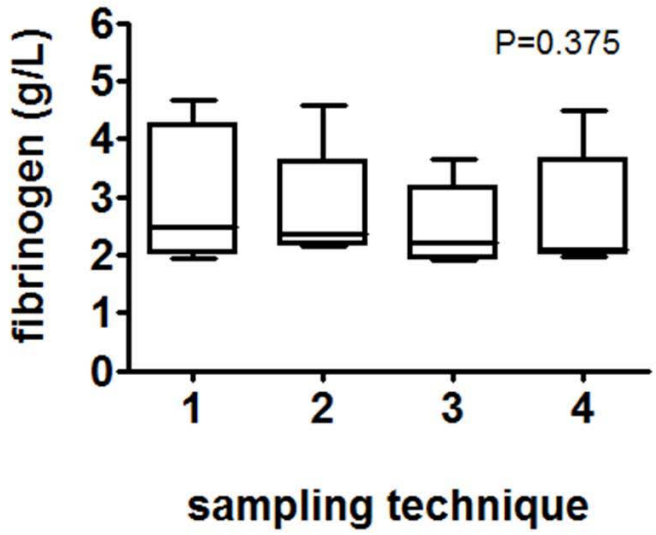

Figure 12: Fibrinogen concentrations which is not significantly affected by the sampling technique (for remainder key, refer to figure 5)

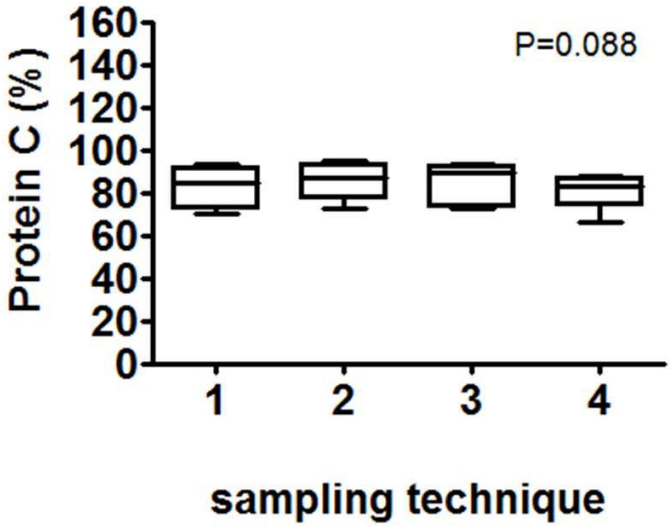

Figure 13: Box and whisker diagram showing no significant changes in protein C concentrations depending on the method of sample acquisition (for remainder key refer to figure 5)

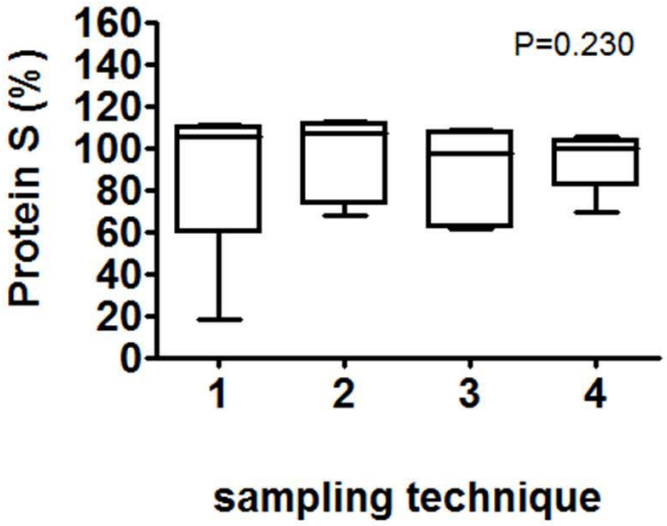

Figure 14: Box and whisker diagram showing no significant changes in protein S concentrations in regard of the sample technique (for remainder key refer to figure 5)

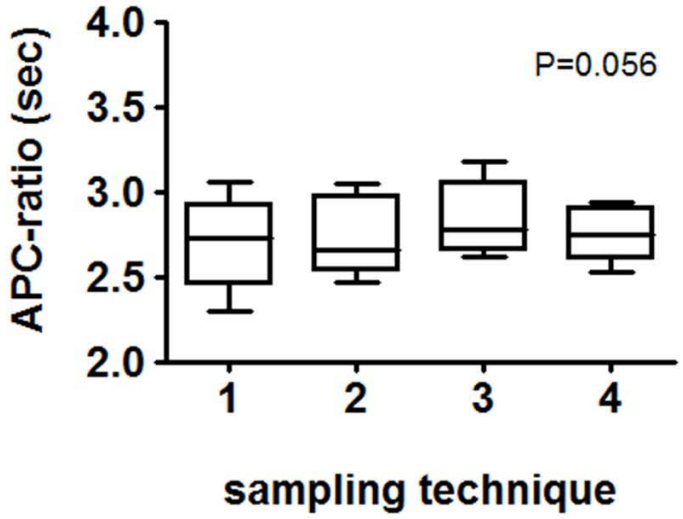

Figure 15: The APC ratio which was not significantly affected by the sampling technique (for remainder key refer to figure 5)

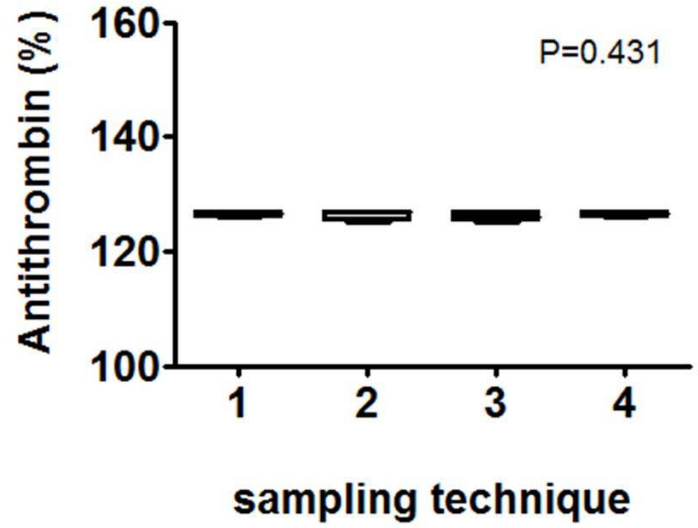

Figure 16: AT III values showing no significant changes depending on the method of sampling (for remainder key refer to figure 5)

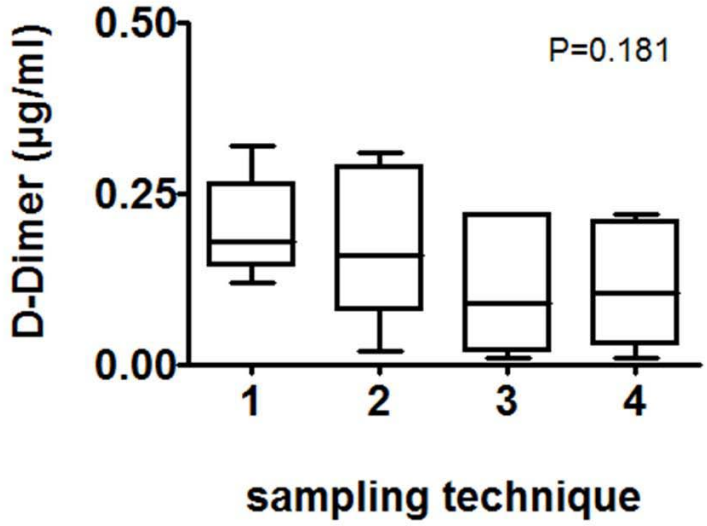

Figure 17: D-dimer concentrations which were not influenced by the sampling technique (for remainder key, refer to figure 5)

3.2 Part 2: Effect of submaximal physical exercise on ADVIA 2120™ platelet activation indices, platelet function, secondary and tertiary hemostasis as well as thrombelastography

The heart rate increased from a median baseline value of 102 beats/min (range 86-132 beats/min) to 192 beats/min (range 131-209 beats/min) directly after the run ($p<0.0001$, figure 18). Heart frequency reached baseline values (median 92 beats/min; range 63-135 beats/min) 60 minutes after exercise. No significant change in lactate plasma concentration directly after finishing running was observed indicative of submaximal exercise (figure 19). The increase in hematocrit from a median baseline value of 0.42 l/l to a median of 0.44 directly after exercise was statistically not significant (figure 20). Postexercise leukocyte count and the number of platelets were not significantly different from baseline values (figure 21, 22).

The PCDW dropped insignificantly from a median of 6.9 g/dL prior to physical activity to a median of 6.1 g/dL ($P=0.0126$) directly after finishing running consistent with a decreased variation in platelet activation status. The number of large platelets dropped significantly from a median number of 28 (range 4-72) to 9 (range 2-63; $P=0.0002$) at time point 13 min. which was also indicative of a decreased platelet activation status after submaximal physical activity whereas the MPM value did not change significantly with a $p=0.3626$. Submaximal exercise was followed by a moderate yet insignificant increase in MPC indicative of decreased platelet activation from a median baseline value of 19.1 g/dL to 21.6 g/dL at time point 13 min. ($P=0.0079$;). There was a significant exercise-induced decrease in MPV ($P=0.0008$) from a median baseline value of 12.3 fL to 10.6 fL following running (figures 23 to 27).

As depicted in figure 28, platelet function reflected by the AUC increased transiently after submaximal exercise. For all agonist concentrations, platelet hyperfunction was followed by platelet hypofunction, but the finding was not significant after Bonferroni correction ($P=0.0092$). A similar tendency was seen for the velocity of aggregation (figure 29). Not

surprisingly, the two way test of variance revealed a significant influence of the agonist concentration on the aggregation AUC and velocity of aggregation.

As shown in figures 30 to 37, there were no statistically significant differences before, after and 60 min. after submaximal exercise in the values of the measured protein C (p=0.430), protein S (p=0.093), FVIII (p=0.152), AT (p=0.656), TT (p=0.839), APC (p=0.043), OSPT (p=0.621) as well as aPTT (p=0.321).

As for the TEG values of the parameters R, K, α, MA and G, there were also no statistically significant differences before, after and 60 min. after submaximal exercise (figures 38 to 41).

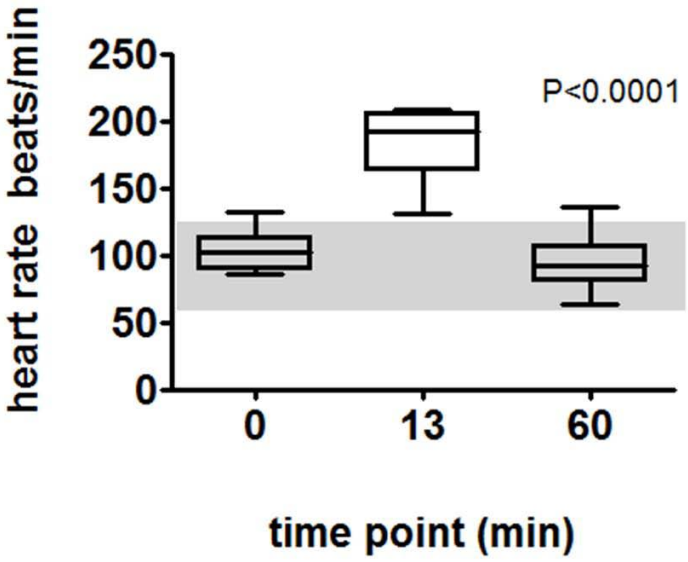

Figure 18: Box and whisker diagram representing the heart rate. The time points were as follows: time point 0 min.: prior to submaximal exercise, time point 13 min.: directly after the run and time point 60 min.: 60 minutes after physical activity; n= 9 dogs.

The central box represents the values from the lower to upper quartile. The middle line is consistent with the median. The horizontal line extends from the minimum to the maximum value. The grey area indicates the reference interval.

*: Statistically significant increase in heart rate at min.13 (The level of significance was set at α = 0.0019 after Bonferroni correction)

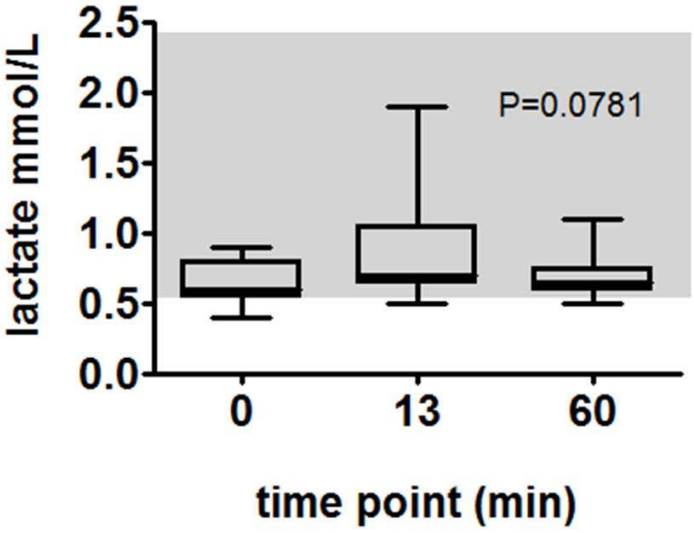

Figure 19: Box and whisker diagram depicting no significant changes between three time points, indicative of submaximal exercise (for remainder key refer to figure 18)

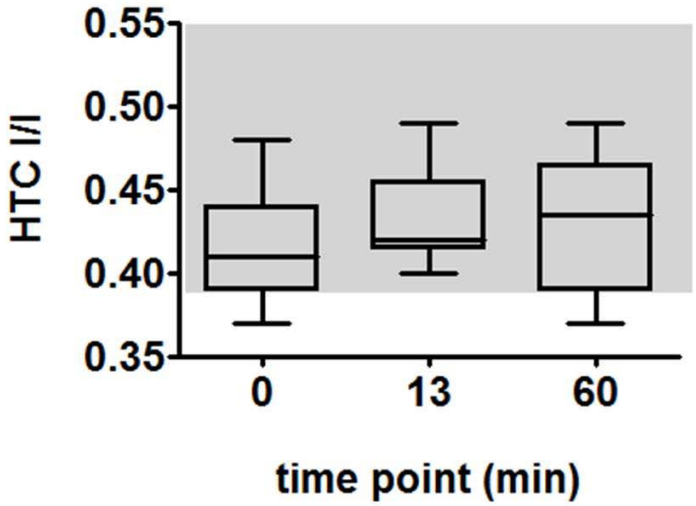

Figure 20: Htc values before, directly after and 60 min. after submaximal exercise (for remainder key, refer to figure 18)

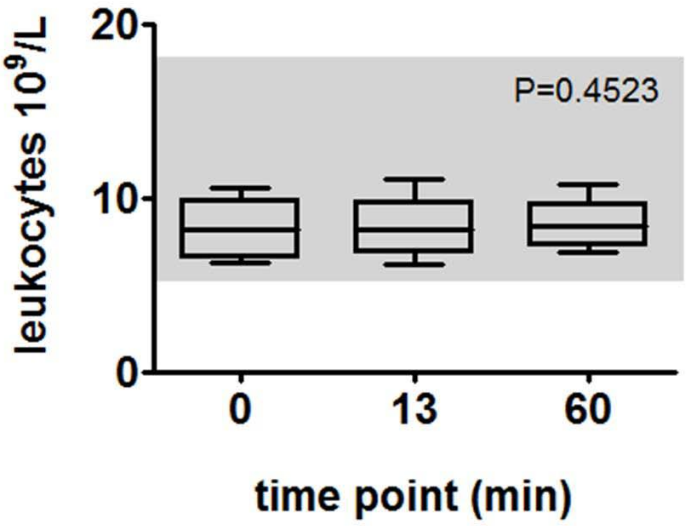

Figure 21: Leukocyte count showed no significant exercise-induced change (for remainder key, refer to figure 18)

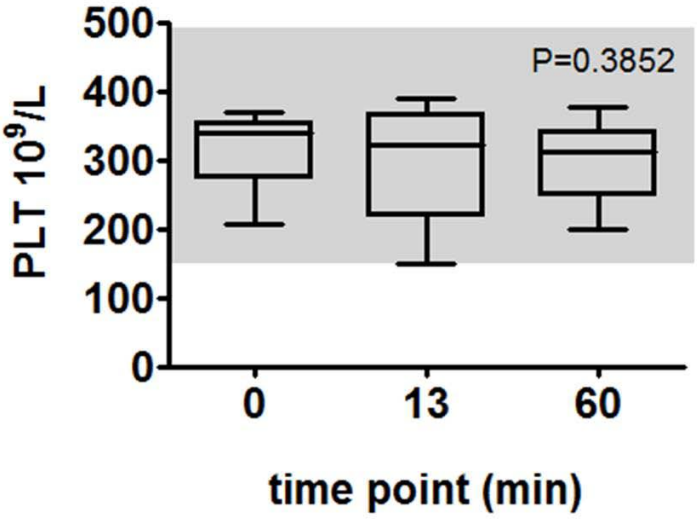

Figure 22: Platelet count was not significantly affected by exercise (for remainder key, refer to figure 18)

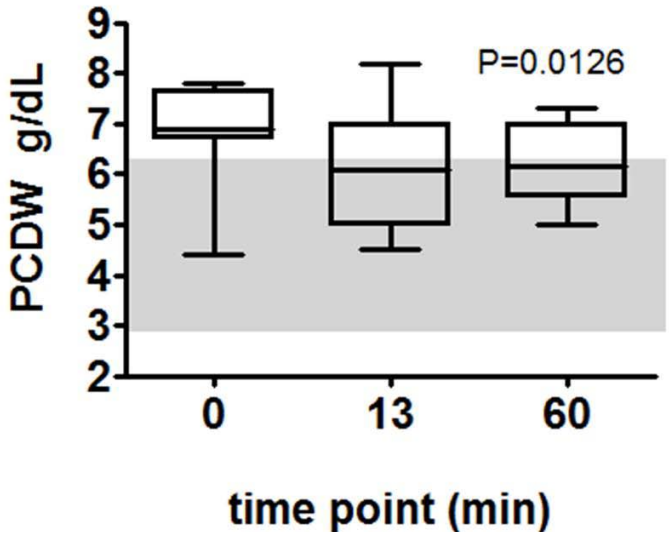

Figure 21:

Box and whisker diagram depeciting the PCDW (platelet component distribution width). (For remainder key, refer to figure 18.)

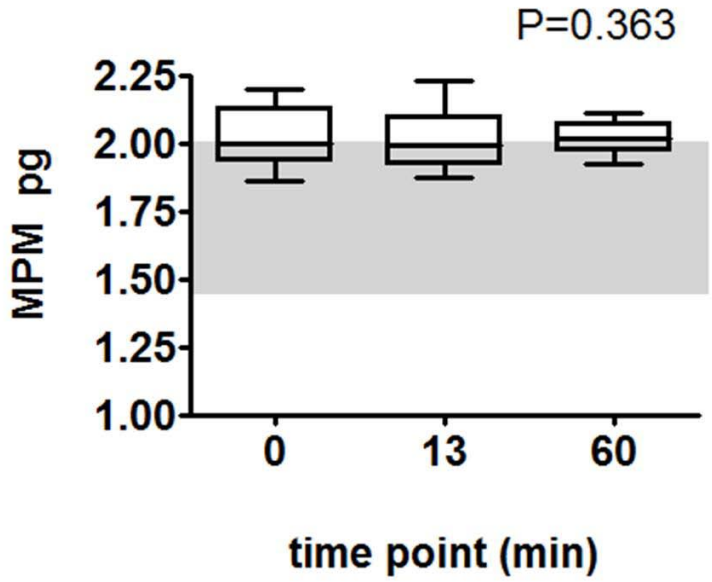

Figure 22:

Box and whisker diagram regarding the (mean platelet mass). (For remainder key, refer to figure 18.)

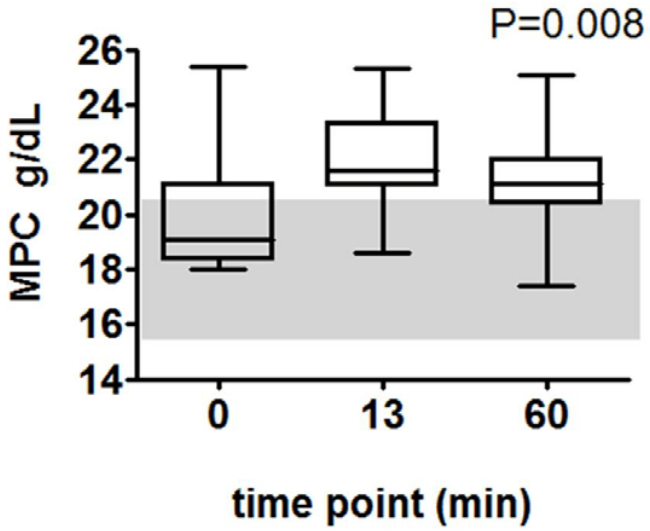

Figure 23: Box and whisker diagrams regarding the ADVIA 2120 platelet activation index MPC (mean platelet component concentration). (For remainder key, refer to figure 18.)

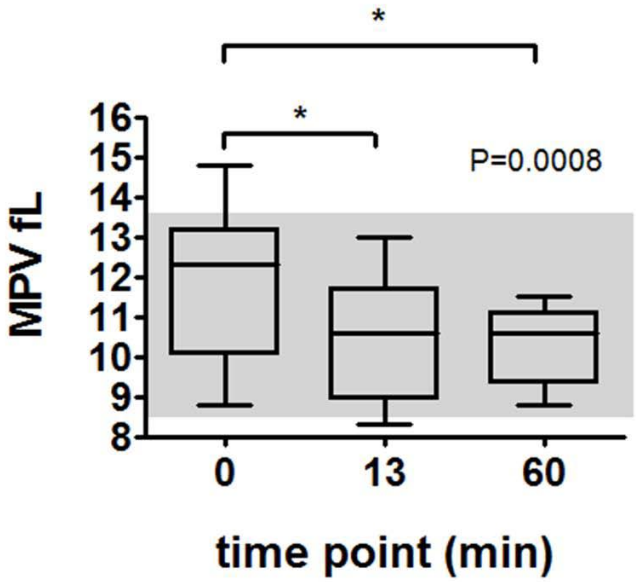

Figure 24: Box and whisker diagrams regarding the MPV (mean platelet volume). (For remainder key, refer to figure 18.)

*: note the significant decrease (p=0.0008)

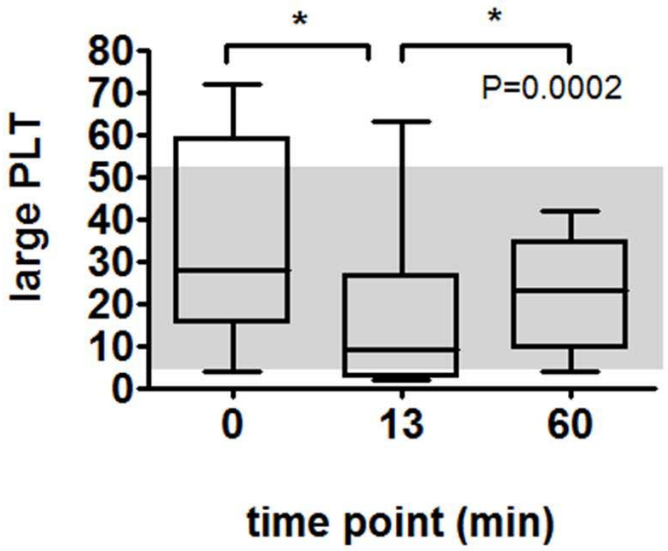

Figure 25: Box and whisker diagram depicting changes in large pletelets before, after and 60 minutes after submaximal exercise, note the significant decrease directly after submaximal exercise (min. 13, p=0.0002)

(for remainder key, refer to figure 18)

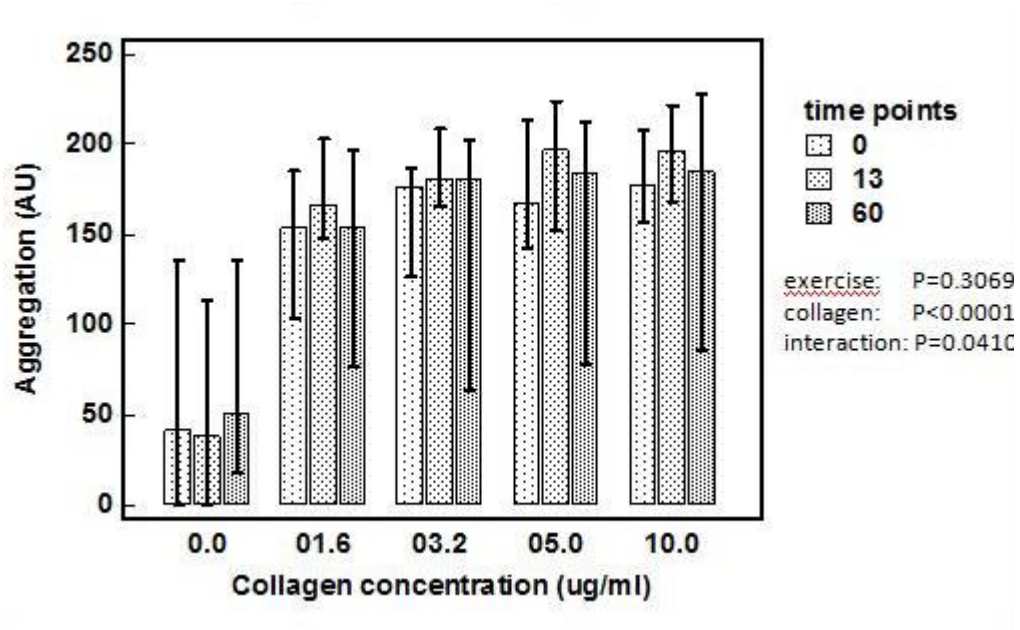

Figure 26:

Box and whisker diagrams showing platelet aggregation (area under the curve) measured with the Multiplate® analyzer prior to and after exercise (For key remainder, refer to figure 18.)

Abbrevations: AU: Aggregation Units

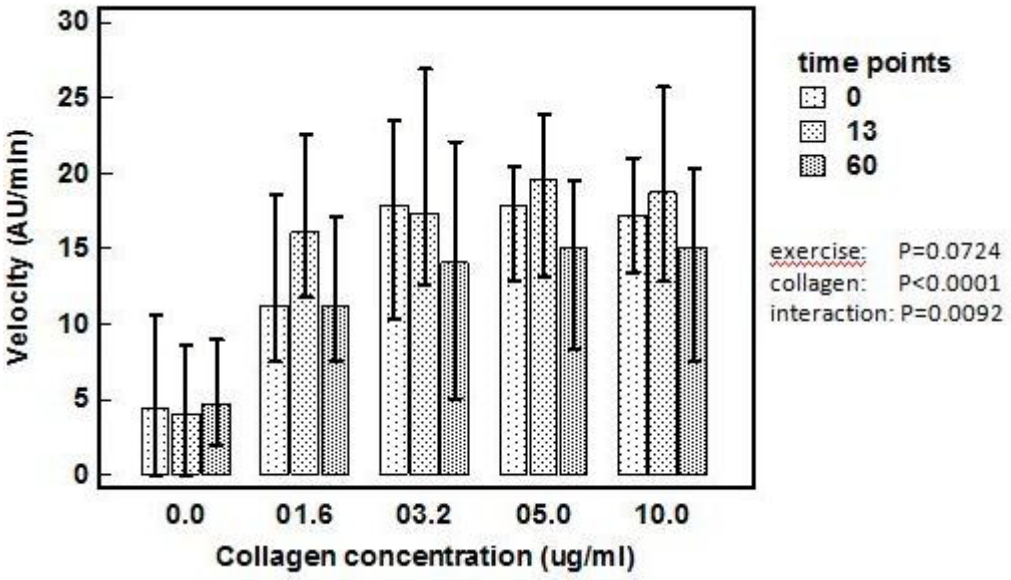

Figure 27:

Box and whisker diagrams showing the impact of exercise on platelet aggregation (velocity) measured with the Multiplate® analyzer (For key remainder, refer to figure 18.)

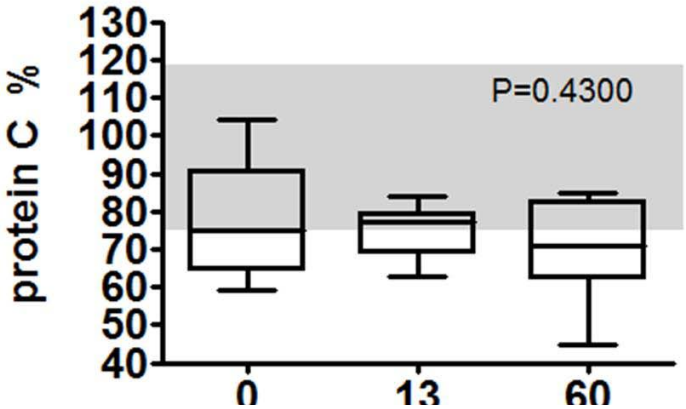

Figure 28: Box and whisker diagram showing no significant exercise-inudced change in protein C concentration (for remainder key, refer to figure 18)

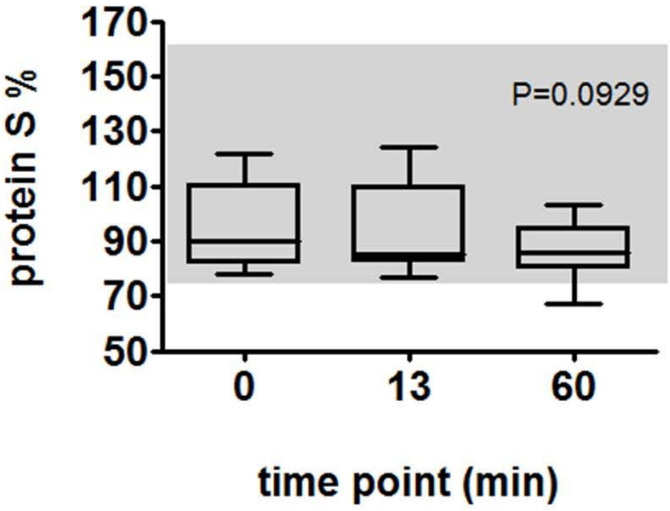

Figure 29: Box and whisker diagram showing no significant change in protein S concentration after submaximal exercise (for remainder key, refer to figure 18)

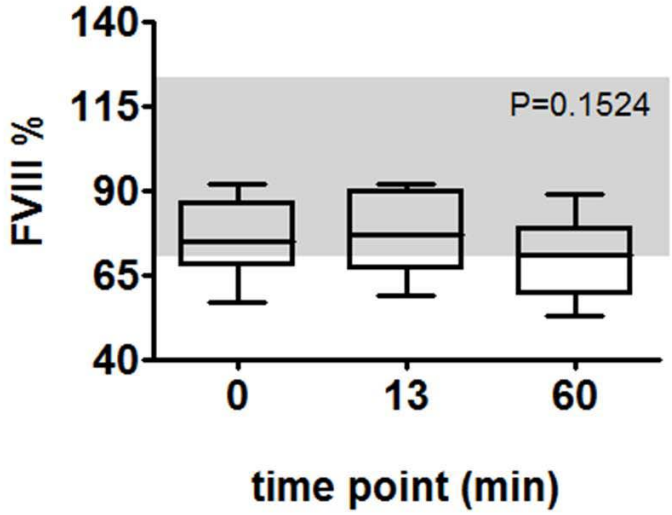

Figure 30: Box and whisker diagram showing no significant exercise induced change in measured factor VIII

(for remainder key, refer to figure 18)

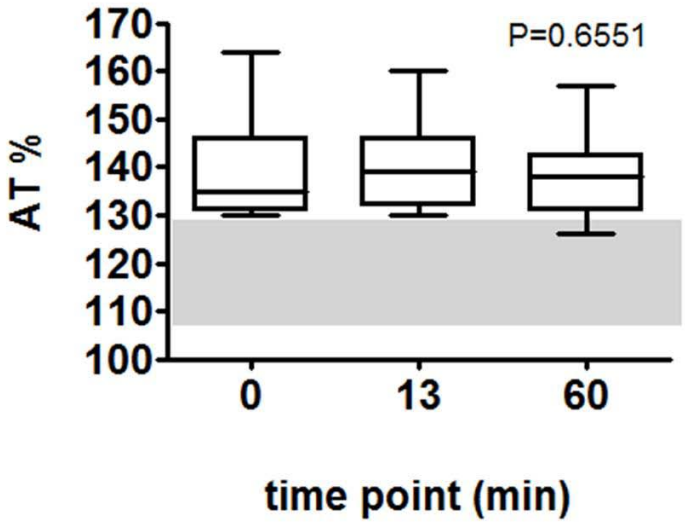

Figure 31: Box and whisker diagram showing measured antithrombin (for remainder key, refer to figure 18)

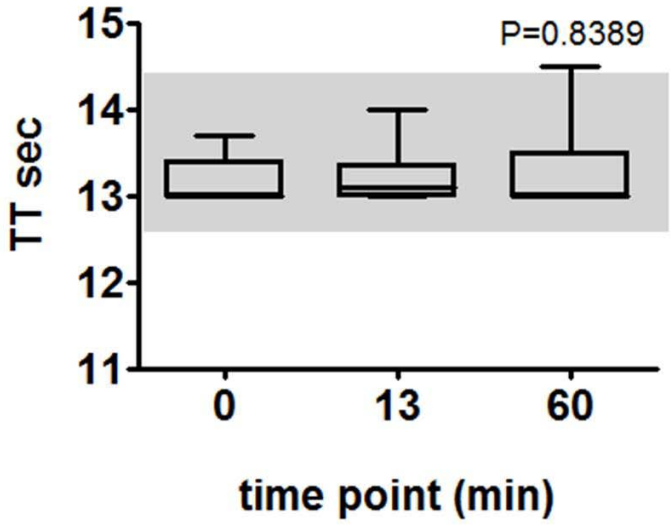

Figure 32: Box and whisker diagram showing thrombin time on three time points (for remainder key, refer to figure 18)

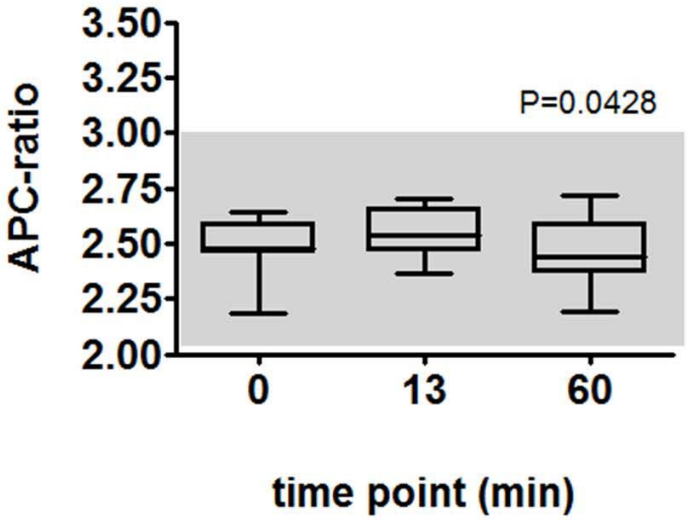

Figure 33: Box and whisker diagram showing no significant exercise induced change in measured activated protein c resistance (APC ratio)(for remainder key, refer to figure 18)

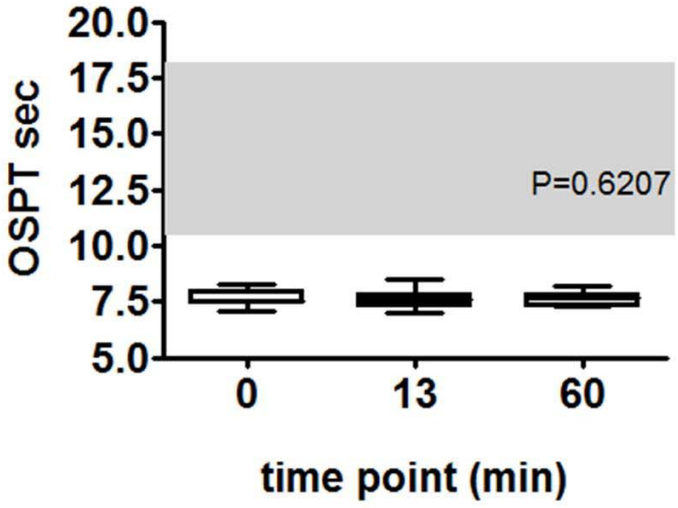

Figure 34: Box and whisker diagram depicting prothrombin time (for remainder key, refer to figure 18)

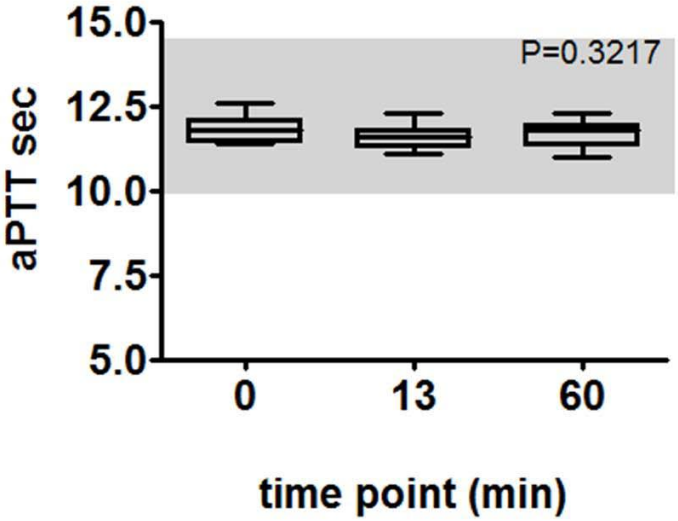

Figure 35: Box and whisker diagram showing measured activated partial thromboplastin time (for remainder key, refer to figure 18)

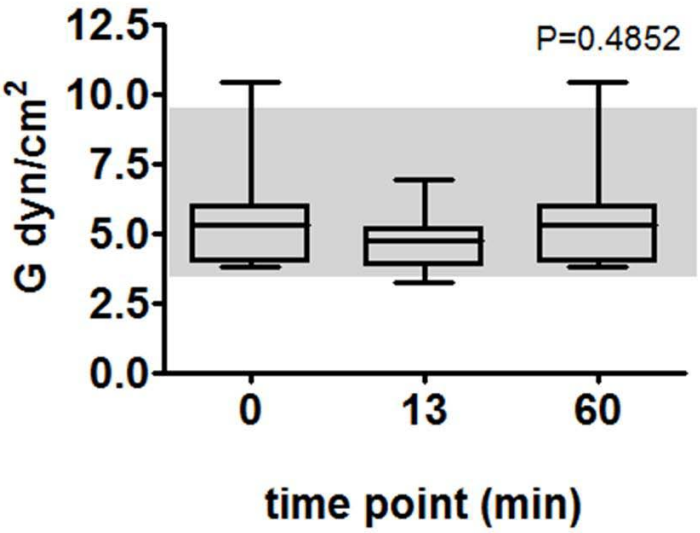

Figure 36: Box and whisker diagram depicting no significant differences in TEG G value after submaximal exercise (for remainder key, refer to figure 18)

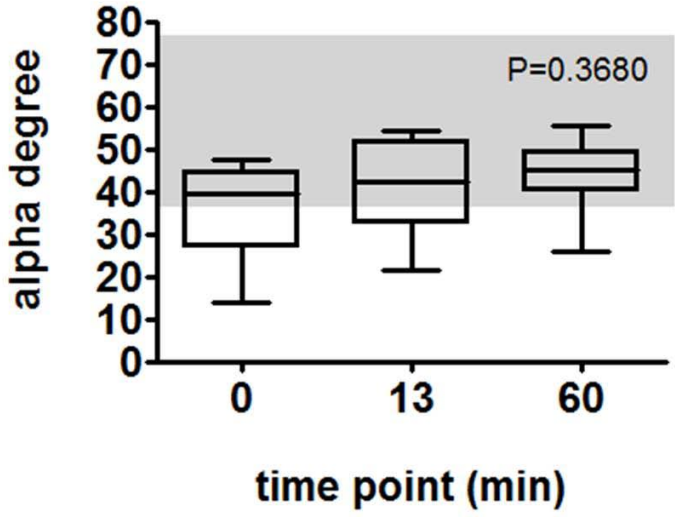

Figure 37: Box and whisker diagram depicting TEG α value

(for remainder key, refer to figure 18)

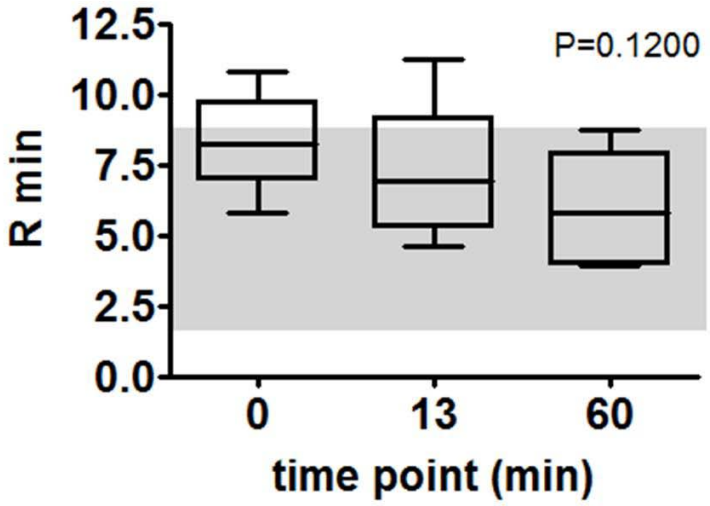

Figure 38: Box and whisker diagram showing TEG R value

(for remainder key, refer to figure 18)

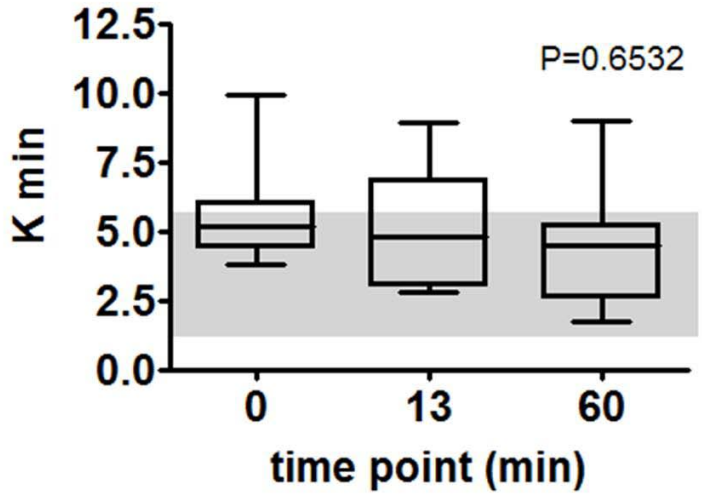

Figure 39: Box and whisker diagram depicting thereis no significant difference in TEG G value

(for remainder key, refer to figure 18)

4 DISCUSSION

4.1 Part 1: Effect of sampling on coagulation variables

A limitation of the current study is the fact that the number of dogs examined was comparatively low so that significant effects might have been missed. Furthermore, only short-term effects were assessed here, although it could be hypothesized that a catheter being in place for a longer period of time might theoretically activate the coagulation system. Further investigations examining the effect of peripheral or jugular catheters being in place for a prolonged time period are warranted.

The pre study which compared the influence of four sampling techniques on primary and secondary hemostasis as well as the inhibitors of coagulation is to the author´s knowledge the first study including measurements to this extent. There are previous investigations in dogs which mainly focused on the comparison of direct venipuncture versus venous catheters (Maeckelbergh et al., 2008, Millis et al., 1995) as well as on the comparison of using venous versus arterial blood for measuring of routine coagulation parameters (Palsgaard-Van Lue et al., 2007). Jugular catheters were compared to venipuncture in one study, whereas others made no differentiation between peripheral and central venous catheters (Maeckelbergh et al., 2008). The pre-study showed that the mentioned venipuncture techniques had no effect on primary, secondary and tertiary hemostasis/fibrinolysis as there were no significant differences.

Although there were no statistically significant changes, the results reported in the pre study might indicate that placing a central venous catheter via the Seldinger technique could induce a slight platelet activation. To our knowledge, there are no similar results which have been reported for dogs before, however, platelet activation and thrombus formation has been intensively studied in coronary animal models as well as in people undergoing coronary catheter interventions. In a sheep coronary model, catheter guide-wires have been reported to cause histologically demonstrable endothelial cell damage, platelet aggregation and thrombus formation which was higher when the guide-wire was manipulated manually (Katsoura et al., 2003). Therefore, it can be hypothesized that insertation of the central

venous catheter with Seldinger technique using a guide-wire might have induced a higher degree of endothelial cell damage and subsequent platelet activation than the over-the-needle technique. Moreover, it has been shown in people that blood collected through catheters introduced with a guide-wire had a much higher plasma heparin neutralizing activity, platelet factor 4 and beta thromboglobulin concentration than peripheral blood (Mant et al., 1984). The evaluation via electronmicroscopy of tips of guide wires used during routine angioplasty procedures in people demonstrated a thrombus formation rate of 26%-80% depending on the type of the guide wire (Gobeil et al., 2002). In a static experiment with human full blood, excessive thrombus formation occurred at heparin-free types of guide-wires whereas models impregnated with sodium-heparin remained essentially thrombus-free (Aldenhoff et al., 2007).

In the dogs evaluated in the pre study, guide-wires used for insertation of central venous catheters with the Seldinger technique were uncoated and catheters were not flushed with heparin or any other compound inhibiting platelet activation so that results obtained for uncoated guide-wires can be compared to the experimental setting in the pre study. On the other hand, the studies in human medicine had a much more invasive approach compared to the relatively non- invasive Seldinger technique used in the pre-study, so that only a limited comparison can be made.

Investigations in people have shown that reduction of the vascular lumen may result in an increased shear stress and - thus, platelet activation. In people, high shear stress is known induce the shedding of procoagulant containing platelet microparticles and therefore contribute to the formation of thrombosis (Miyazaki et al., 1996). Shear stress due to different catheter lumina may contribute for the evidence of platelet activation observed in samples taken via central venous catheters applied with the Seldinger technique as the this catheter type had a smaller lumen (14G) than the central venous catheter placed with the over-the-needle technique (13G). However, shear stress of platelets induced by a small diameter of the needle/catheter cannot be the only cause for higher platelet activation observed in samples taken through the central venous catheter placed with the Seldinger

technique as the highest activation of platelets would have been expected in specimens collected via direct venipuncture with a 20G needle or through 18G catheters.

The impact of sampling technique and lumen of the catheter/needle on routine coagulation test has been rarely reported for dogs. A recent study compared the effect of arterial versus venous sampling on additional variables i.e., factor VIII, protein C and antithrombin and could not demonstrate any significant difference between the results (Palsgaard-Van Lue et al., 2007). However, different techniques of venous sampling were not investigated. To the author's knowledge fibrin D-dimers, protein S, and APC-ratio have not been evaluated for both animals and people. In patients, several pre-analytical factors are known which might influence results of routine coagulation parameters (PT, aPTT) such as the size of the catheter, local activation of coagulation in catheters remaining in the vein for more than one hour, sampling near a catheter through which anticoagulatory therapy was applied and hemodilution of the sample resulting from medication and intravenous therapy (Laxson et al.,1994, Lindley et al.,1994, Molyneaux et al.,1987, Reinhardt et al.,1987). The last two factors can be ruled out for the pre study. Different sizes of catheters were used here without having any significant impact on coagulation parameters.

Regarding secondary hemostasis, the results detailed here are in accordance with a previous study in 14 clinically healthy dogs demonstrating no significant difference in PT, aPTT, fibrinogen concentration and fibrin degradation products in samples obtained from either 16G jugular catheters or by venipuncture (diameter of the needle was not specified) (Millis et al.,1995). In contrast to the current pre study, jugular catheters were heparinized, however, a two syringe method was used for sampling and the first volume of blood was discarded to avoid any effect of heparin on coagulation parameters (Millis et al., 1995).

These findings and the current results, a recent study in dogs hospitalized in an intensive care unit demonstrated that the aPTT determined in blood samples drawn by venipuncture (reference group) with a 21G needle was significantly shorter than results obtained by sampling with either a 20G or 18G catheter (experimental group, $p<0.05$) when the samples were taken at the first day of receiving the catheter (Maeckelbergh et al., 2008). However, the difference between aPTT results determined in the reference group and the

experimental group was small and therefore not considered clinically relevant despite the statistical significance (Maeckelbergh et al., 2008). Moreover, using a 22G catheter, did not have a significant impact on aPTT (Maeckelbergh et al., 2008).

Although rarely investigated in dogs, the influence of sampling technique on routine coagulation parameters was subject of several studies in people. In agreement with the current results, measurements of PT and aPTT in 120 patients under anticoagulation therapy which were either performed in samples drawn through a 21-gauge needle (venipuncture method) or 18G, 20G, 22G peripheral venous catheters (peripheral venous catheter method) were statistically equivalent (Zengin et al.,2008). A similar result was obtained in six patients with hemophilia A under non-bleeding conditions for aPTT, PT and FVIII comparing peripheral venipuncture with sampling through a venous catheter (Lindley et al., 1994).

It was the aim of the current study to investigate the sole effect of sampling technique on coagulation parameters so that heparinization of the catheters was strictly avoided. However, several clinical studies in people investigated the influence of sampling via a heparinized central venous catheter. If the blood was directly taken out of the catheter, a significant prolongation of aPTT could be observed. The effect, however, was not evident if more than the 6-fold of the catheter filling lumen was discarded as shown in an integrative retrospective study in people (Laxson et al., 1994).

Based on the results of the pre study it can be concluded that the sampling technique did not have any significant influence on variables reflecting the coagulatory state so that various sampling techniques can be used. However, sample taking via central venous catheters placed with Seldinger technique might induce platelet activation which should be considered if platelet function is of interest.

4.2 Part 2: Effect of submaximal physical exercise on ADVIA 2120™ platelet activation indices, platelet function, secondary and tertiary hemostasis as well as thrombelastography

In the actual study, submaximal exercise was associated with a significant decrease in large PLTs and MPV indicative of decreased platelet activation status. A possible explanation for this might be the consumption of large platelets due to increased platelet activation directly after exercise, thus, the decrease in MPV. Interestingly, the previous study in dogs exhibited a decrease in the ADVIA 120™ platelet activation indices MPC and PCDW suggestive of platelet activation after short duration strenuous sled-pulling activity (Moritz et al., 2003). A similar result was observed in athletes after finishing a marathon run. In contrast to the current investigation, however, the effect of strenuous rather than submaximal exercise has been evaluated in the latter two studies which resulted in leukocytosis (Kratz et al., 2006) and activation of neutrophils (Moritz et al., 2003) most likely due to an inflammatory reaction caused by exercise-induced tissue damage. In the dogs evaluated here, there was no evidence of an inflammatory reaction, also a strenuous exercise was not the case as plasma lactate levels were not elevated after exercise, which is a probable explanation for the difference found between the current study and the previous investigation. In dogs, it is well known that inflammatory reaction alone is associated with platelet activation when assessed by ADVIA 120/2120™ platelet activation indices (Moritz et al., 2005). It can be hypothesized that the postexercise decrease in platelet activation might be a physiological counter-regulation following previous platelet activation during physical activity. The latter can be explained by epinephrine-effect and increased shear stress which are known to activate platelets synergistically through von Willebrand factor (vWF) interaction to glycoprotein (GP) Ib (Goto et al., 1996). In humans, it is well known that platelet activation is followed by degranulation, transient aggregate formation mediated by vWF and finally de-aggregation resulting in re-circulation of exhausted defective platelets with secondary storage pool disease (Michiels et al., 2006). This mechanism is a probable explanation for the exercise-induced presence of hypoactive platelets.

As reviewed previously, exercise-induced hypercoagulability is well known in humans performing acute and strenuous exercise, particularly for untrained individuals (Lippi et al., 2009). Given the data from human studies, the training status of the individuals has an important impact on the hemostatic system as hypercoaguable state and subsequent exercise-induced thromboembolic complications or sudden death during and immediately after the exercise were especially observed in untrained persons (Lippi et al., 2009). The dogs included in the current study were not specifically trained so that a higher prethrombotic risk could be expected here than in dogs trained for dog sports, racing etc.

Given the negligible increase in lactate, the type of exercise was submaximal and therefore lower than in the majority of human studies (Lippi et al., 2009) so that results are not entirely comparable. Even in people, unequivocal conclusions about the influence of coagulation on the hemostatic system are not possible as the available studies in the literature are influenced by the subjects investigated, the type, intensity and duration of exercise as well as the laboratory methods used for assessment of hemostasis (Lippi et al., 2009). Submaximal exercise was chosen here for ethical reasons as conditioning dogs to run on the treadmill is difficult for maximal exercise resulting in extreme exhaustion. Moreover, the majority of pet-dogs are rarely submitted to maximal exercise, i.e. physical activity exceeding the aerobic threshold, so that this experimental study is likely to provide clinically useful data for the majority of family dogs presented in veterinary practices and clinics.

As reviewed elsewhere, hypercoagulable state in people is caused by an increased FVIII activity, vWF as well as platelet hyperreactivity (Lippi et al., 2009). The latter etiology is consistent with the findings in the current study and with a previous investigation reporting an increased activatability of platelets in sled dogs when exposed to *phorbol myristate* acetate (PMA) (Moritz et al., 2003). In accordance with the current investigation in dogs, human platelets demonstrated a markedly increased sensitivity to collagen-induced aggregation in amateur runners taking part in a marathon race (Dimitriadou et al., 1977). In contrast to this, markedly decreased platelet function was observed previously in marathon runners directly after the race and 24 hours after finishing strenuous physical activity (Rock et al., 1997).

However, other than in the human investigation, there was no increased FVIII activity in dogs. Studies in healthy volunteers demonstrated that activation of platelets in response to exercise could not be prevented by use of platelet aggregation inhibitors aspirin or clopidrogel which was partially attributed to the concurrent exercise induced raise of plasma vWF seen in these individuals (Hjorth et al., 2009).

Though not significant, TEG variables in the dogs evaluated here demonstrated a tendency towards activation of secondary hemostasis reflected by a decrease in R and K as well as an increased angle α. This finding was in accordance with a human study evaluating ROTEM thromboelastography in amateur runners after a 42,195 m downhill race. After the run, a shortening of the intrinsic pathway clotting time (comparable with the TEG R-value although kaolin represents the extrinsic pathway) and the clot formation time (comparable with the TEG K-value) as well as an increase in α-angle was reported (Sumann et al., 2007). However, in contrast to humans exhibiting an increase of the maximum clot firmness (comparable with TEG-MA value) following 42,195 m downhill race (Sumann et al., 2007), MA was not influenced in the dogs evaluated here. A probable reason for this is the fact that a downhill marathon race is much more exhausting than the submaximal exercise performed in the current study. As muscular microtrauma was considered to be responsible for the activation of the coagulation system (Fehrenbach et al., 2006), it can be hypothesized that activation of coagulation increases with the severity of physical exercise.

This is also a probable reason for the fact that a marked activation of coagulation reflected by coagulation times aPTT, OSPT, FVIII as well as an increase in natural inhibitors of coagulation including protein C, protein S and evidence of fibrinolysis indicated by an increase in D-dimers was seen in humans after strenuous exercise (Fehrenbach et al., 2006) but not in the dogs evaluated here.

Regarding the methodology used in the current study, sample acquisition was performed through a central venous catheter inserted with “catheter-through the needle” technique to provide a rapid sampling at the exact time points and to avoid pain or excitement of the dogs which might influence the results. Sampling through venous catheters may theoretically induce shear stress and thus changes of the coagulation pattern. However, a

sampling-induced influence of coagulation was ruled out previously for this type of jugular catheter used also in the current study (Part I, of this thesis work, Bauer et al., 2010).

A novel impedance-based whole blood aggregometer was chosen to assess platelet function due to the fact that light transmission aggregometry (Born method) - the most commonly used method for platelet function testing - has several drawbacks. Disadvantages of the Born method include the need of preparation of platelet rich plasma (PRP) resulting in a separation of other blood cells from platelets which are also known to influence platelet function (Santos et al., 1991, Bartlett et al., 1977), the loss of platelets during the process of preparation as shown in people (Persidsky et al., 1982, Reiss et al., 1976, Hill et al., 1988) and the fact that giant platelets which may be both hypo- or hyperactive are commonly not included in PRP (Dyszkiewicz-Korpanty et al., 2005).

The impedance aggregometer used in the current study determines platelet function in diluted whole blood by using disposable test cells with duplicate impedance sensors. Thus, possible sources of error such as cleaning of electrodes between analyses which had to be performed in older impedance aggregometers were avoided (Toth et al., 2006). In the current investigation, the thrombin antagonist hirudin was chosen as an anticoagulant as previously recommended for dogs because it is known to preserve the physiological concentration of ionized calcium and magnesium better than citrate (Kalbantner et al., 2008). Like in the previous investigation in dogs, agonist concentrations starting with 10µg collagen/ml were chosen as here the lowest intra-assay variation was observed (Kalbantner et al., 2010).

In the current study, kaolin has been used as an agonist for TEG analysis. Other investigators performed TEG in canine specimens without any inductor (Vilar et al., 2008) or with the activator tissue factor (TF) (Wiinberg et al., 2005). Kaolin was chosen as an activator here because laboratory-intern reference intervals have been established for this method so that the results could be interpreted in relation to the reference range. Another reason was the fact that a lot of clinical studies use an activator of the coagulation process to simulate the *in vivo* process of coagulation. Tissue factor has been considered the best activator for this purpose (Sorensen et al., 2003) but it is not available in ready-to use vials so that it is less

likely to be used in a routine clinical laboratory. Moreover, tissue factor has to be diluted from a stock solution so that there is a higher probability of pre-analytic errors (Lippi et al., 2007, Laposata et al., 2007).

Regarding the preparation of citrated plasma, it has to be taken into account that the centrifugation speed routinely used in the author's laboratory was lower than 1500g, i.e. the force recommended by the Clinical and Laboratory Standards Institute (CLSI) (Adcock et al., 2008). However, according to the CLSI recommendations, alternate times and forces may be used as long as the plasma platelet count is $\leq 10 \times 10^9/L$[46] which has been demonstrated to be achieved by the protocol used in the current study. Even for human laboratories, a huge variation in centrifugation force was reported ranging from 500-3000g (Pappas et al., 1991).

A limitation of the study was the fact that the number of dogs examined was comparatively low so that significant effects might have been missed.

Based on the results of the current study, it can be concluded that submaximal exercise did not have a major impact on the coagulation system and is therefore not associated with a thromboembolic risk. Although the thromboembolic risk appears minimal in healthy dogs, it should be considered in patients with underlying diseases associated with a hypercoagulable state and further studies comparing different exercise types in healthy dogs are needed.

5 SUMMARY

In summary, in the first part of the study the impact of four different sampling techniques (20G intradermic needle, 18G venous catheter, a 14G x 16 cm radiopaque polyurethane central venous catheter inserted with Seldinger technique and a 13G central venous catheter placed with the “over-the-needle” method) on the primary hemostasis (whole blood aggregation) and coagulation parameters PT, aPTT, fibrinogen plasma concentration, FVIII activity, natural inhibitors of coagulation (AT, PC, PS, APC-ratio) and fibrin D-dimer plasma concentration as well as a kaolin-activated TEG analysis as a global test was investigated . There were no statistically significant changes in the previously described markers.

In the second part of the study, the effect of submaximal exercise on whole blood aggregation, ADVIA 2120 activation indices, PT, aPTT, fibrinogen plasma concentration, FVIII activity, natural inhibitors of coagulation (AT, PC, PS, APC-ratio) and fibrin D-dimer plasma concentration as well as a kaolin-activated TEG analysis as a global test before, directly after and 60 minutes after exercise was examined.It was observed that directly after submaximal exercise, a decreased platelet activation state was present. A subsequent mild transient postexercise platelet hyperreactivity with a following platelet hypofunction was also noticed.

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