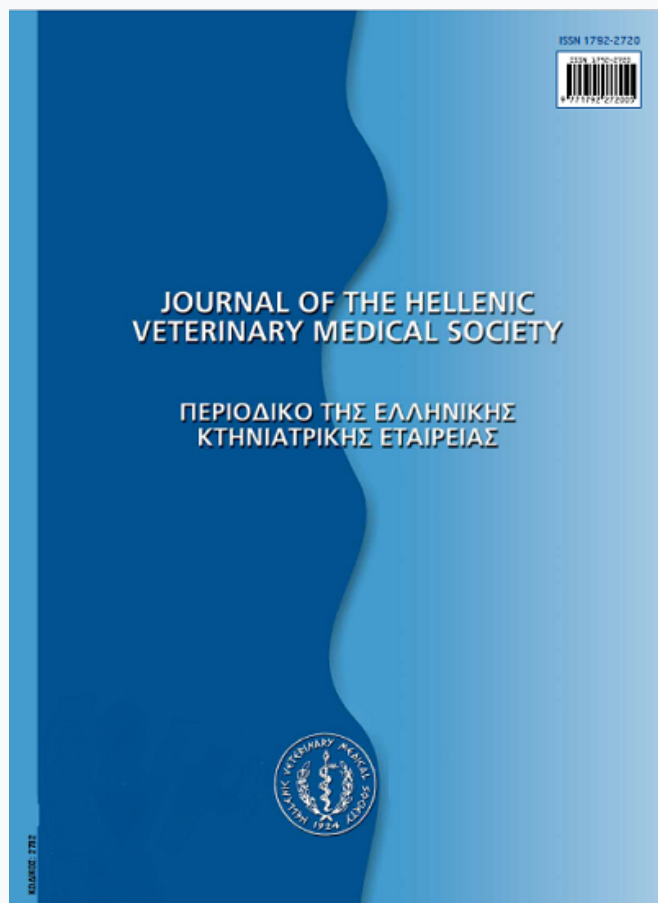


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### Laboratory evaluation and interpretation of haemostasis in small animals

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## **Laboratory evaluation and interpretation of haemostasis in small animals**

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## **Η εργαστηριακή διερεύνηση της αιμόστασης στα ζώα συντροφιάς**

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### **ABSTRACT**

This review deals with indications for performing haemostasis tests, methodical aspects of blood sampling and handling of sample material, selection of appropriate tests and basic principles of test interpretation. Indications for laboratory evaluation of haemostasis in small animals include spontaneous bleeding or bleeding disproportionate to the degree of trauma, bleeding from multiple sites and diseases which are frequently associated with haemostatic disorders. Patient history and clinical findings can deliver clues for the type of haemorrhagic diathesis. Important preanalytical issues include prevention of prolonged venous stasis, order of drawing and appropriate filling of the collection tubes and careful mixing of blood with anticoagulant.

Initial screening can be performed by global tests of haemostasis including viscoelastic testing using thrombelastography and rotation thrombelastometry and/or a “basic examination profile” including platelet count, capillary bleeding time (optional in cases of suspected functional platelet disorders) and group tests prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin (clotting) time (optionally in cases of prolonged PT and APTT). This procedure allows a rational evaluation of the haemostatic system and a specific use of further tests including individual coagulation factor activities, inhibitor activities, activation markers, examination of von Willebrand factor, and platelet function analyses. It is important (1) to use species- and method specific reference values for interpretation and (2) to consider the limitations of tests when performed according to the standard test procedure optimised for human sample material (e.g., low sensitivity of PT test for canine and feline samples).

### **Keywords:**

Blood coagulation, individual coagulation factors, platelet count, platelet function analysis, preanalytics, screening test.

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## ΠΕΡΙΛΗΨΗ

Στην ανασκόπηση αυτή αναφέρονται οι ενδείξεις για τον εργαστηριακό έλεγχο της αιμόστασης, η επιλογή των κατάλληλων για κάθε περίπτωση εξετάσεων, η μεθοδολογία για τη λήψη και το χειρισμό των δειγμάτων και η ερμηνεία των αποτελεσμάτων. Οι συνηθέστερες ενδείξεις για τον έλεγχο της αιμόστασης είναι η αιμορραγική διάθεση και η διάγνωση νοσημάτων που συνοδεύονται από διαταραχή της πήξης του αίματος. Σε αρκετές περιπτώσεις η ανασκόπηση του ιστορικού σε συνδυασμό με τα ευρήματα της κλινικής εξέτασης παρέχουν χρήσιμη πληροφόρηση για την αιτιολογία της αιμορραγικής διάθεσης. Κατά τη δειγματοληψία θα πρέπει να αποφεύγεται η παρατεταμένη ίσχαιμη περιδέση και να επιλέγονται τα φιαλίδια με το κατάλληλο αντιπηκτικό που θα γεμίζονται αυστηρά με την προκαθορισμένη κατά περίπτωση ποσότητα αίματος. Η ανάμιξη του αίματος με το αντιπηκτικό θα πρέπει να γίνεται με ήπιους χειρισμούς. Η εργαστηριακή αξιολόγηση της αιμόστασης περιλαμβάνει την μέτρηση του αριθμού των αιμοπεταλίων, του χρόνου ροής (προαιρετικά όταν υπάρχει υποψία θρομβοκυτταροπάθειας), του χρόνου προθρομβίνης (PT), του χρόνου ενεργοποιημένης μερικής θρομβοπλαστίνης (APPT), του χρόνου πήξης (προαιρετικά, όταν οι PT και APPT είναι αυξημένοι) και το θρομβοελαστόγραμμα. Η μεθοδική αυτή αξιολόγηση της αιμόστασης μπορεί να επεκταθεί με την εφαρμογή πιο εξειδικευμένων εξετάσεων όπως η μέτρηση των παραγόντων πήξης του αίματος, ο προσδιορισμός του παράγοντα von Willebrand και οι λειτουργικές δοκιμασίες των αιμοπεταλίων. Για την αξιοπιστία των ελέγχου της αιμόστασης θα πρέπει (1) να χρησιμοποιούνται μέθοδοι που έχουν αξιολογηθεί για εφαρμογή στο ζωικό είδος που εξετάζεται και (2) να λαμβάνονται υπόψη οι περιορισμοί που ισχύουν όταν εφαρμόζονται στα ζώα μέθοδοι που προορίζονται για τον άνθρωπο (για παράδειγμα, η μέθοδος μέτρησης του χρόνου προθρομβίνης στον άνθρωπο δεν είναι αρκετά ευαίσθητη για χρήση στο σκύλο και τη γάτα).

**Λέξεις ευρετηρίασης:** αιμοπετάλια, αιμόσταση, γάτα, θρομβοκυτταροπάθεια, παράγοντες πήξης αίματος, σκύλος,

## INTRODUCTION

Adequate haemostasis depends on normal structure and function of the blood vascular system, platelet numbers and normal platelet function, and an adequate coagulation system. The vascular component can rarely be diagnosed as cause of haemostatic disorders, because standardised tests for vascular haemostatic dysfunction do not exist in veterinary medicine. In contrast, haemostatic disorders due to either quantitative and qualitative platelet defects or coagulation system disorders can be defined in detail by numerous specific tests.

A detailed history and a thorough clinical examination can deliver clues for an underlying haemostatic disorder and its type (Hackner, 1995; Herring and McMichael, 2012). Bleeding patterns can indicate the cause of haemorrhage diathesis. Mucosal and cutaneous petechiae, purpura and ecchymoses are characteristic findings in disorders of primary haemostasis (thrombocytopenia, thrombocytopathia) (Figure 1) (Botsch et al., 2009; Wondratschek et al., 2010; O'Marra et al., 2011). In contrast, blood coagulation disorders such as haemophilia are associated with



**Figure 1.** Petechiae and ecchymoses in the pinna of a cat suffering from severe thrombocytopenia.

**Table 1.** Indications for laboratory evaluation of haemostasis in small animals

- Haemorrhages, swellings and body cavity effusions of uncertain origin
- Underlying diseases which are frequently associated with haemostatic disorders (e.g. liver diseases, haemangiosarcoma)
- Monitoring of known haemostatic disorders
- Control of anticoagulant and fibrinolytic therapy
- Preoperative screening
- Breeding programmes

larger areas of haemorrhage, e.g. subcutaneous or intramuscular haematomas, body cavity and joint haemorrhages (Mischke, 2013) (Figure 2). Bleeding from mucosal surfaces (e.g., epistaxis, oral cavity haemorrhage, haematemesi, haematuria, haematochezia, melaena) can be associated with disorders of primary or secondary haemostasis.

Global tests and/or a basic haemostasis examination profile with screening tests enable a rational assessment of the haemostatic function and, in case of a haemostatic disorder, in many cases allow already to define its type or indicate specific tests necessary for final diagnosis (e.g., factor VIII and IX measurements for haemophilia diagnosis).

## INDICATIONS FOR HAEMOSTASIS TESTING

Spontaneous bleeding (without detectable cause), bleeding disproportionate to degree of trauma



**Figure 2.** Severe bleeding in the oral cavity and retropharyngeal and ventral neck region of a German Shepherd dog with severe haemophilia A.

and bleeding from multiple sites are suspicious for haemostatic disorders and, therefore, are among the indications to perform laboratory tests of haemostasis (Table 1).

## SAMPLE MATERIAL

### Collection and handling

Haemostasis tests make higher demands on blood sampling techniques than most other parameters in the clinical laboratory (Lippi et al., 2012). For the measurements of platelet counts, K-EDTA blood is required. Blood coagulation tests are performed with plasma prepared from blood anticoagulated with sodium citrate or citrate buffer. For whole blood platelet aggregation studies (with the Multiplate analyser) hirudin-anticoagulated blood shows better results (Kalbantner et al., 2010).

### Sample tubes for citrated blood

To obtain citrated plasma, prefabricated sample containers are available from various manufacturers. The sample containers are prefilled with an adequate volume of 0.11 mol/l sodium citrate or citrate buffer solution to guarantee a mixture ratio of 9 parts of blood and one part of anticoagulant, when tubes are filled up to the graduation with blood. This results in a ratio between plasma and citrate solution of approximately 4.5 to 1 in a sample with normal haematocrit (e.g., 50 %). Underfilling of the vials by more than 10 % is critical, because human studies demonstrate that this leads to significantly prolonged coagulation times, especially of the activated partial thromboplastin time (APTT) (Reneke et al.,

**Table 2.** Combined interpretation of test results from routine tests of haemostatic function and recommended further tests to differentiate between differential diagnoses

<b>Platelet count</b>	↓ – ↓↓↓	→	→ / ↓	↓↓	→	→ / ↓
<b>Bleeding time</b>	→ / ↑ – ↑↑	↑ – ↑↑↑	→	→	→	→
<b>PT</b>	→	→	↑↑↑	↑↑	↑	→ / ↑
<b>APTT</b>	→	→	↑↑	↑↑↑	↑	↑ – ↑↑
<b>Thrombin time</b>	→ / ↓	→	↓ / →	↑↑	→ / ↓	→ / ↓
<b>Diagnosis / Differential diagnosis</b>	Thrombocytopenia	-vWD -Platelet dysfunction	Anticoagulant rodenticide intoxication	DIC (final stage)/ hyperfibrinolysis	-DIC -Liver induced -Vitamin K-dependent	-Haemophilia -Contact factor deficiencies -DIC, etc.
<b>Further recommended tests to confirm diagnosis</b>	—	-vWF -Collagen binding activity -Platelet function tests	Control of therapeutic effect after vitamin K <sub>1</sub>	—	-Individual coagulation factors (-Activation markers)	Individual coagulation factors

PT – Prothrombin time; APTT – activated partial thromboplastin time; vWD – von Willebrand's disease; vWF – von Willebrand factor; DIC – Disseminated intravascular coagulation

↓ – ↓↓↓ Mild to severe decrease/shortening; ↑ – ↑↑↑ Mild to severe prolongation  
→ Normal finding; ↓ Reactive shortening; / or (alternative finding)

1998; Lippi et al., 2012).

Controlled over- or underfilling, respectively, of the tubes with blood may be indicated to achieve an adequate mixture ratio between the plasma content and citrate solution, when the haematocrit is markedly increased or decreased (e. g., the ratio between plasma and citrate solution is approximately 2.25 to 1 in a sample with a haematocrit of 75 %, whereas overfilling of the tube to approximately 190 % of the declared volume results in an adequate ratio).

### Blood collection

Blood collection should be carried out, if possible, without raising the vein or performing this only slightly and briefly (< 30 s) to avoid significant activation of the haemostatic and fibrinolytic system (Lippi et al., 2012). A sharp disposable needle with an adequate lumen (e. g., 1.1x30 mm [19G] for dogs) should be used. The first drops of blood should be discarded or used for other sample tubes to avoid contamination of the samples with tissue factor, because this may result in misleading results

due to activation of clotting factors and/or platelets. The blood should flow carefully along the wall of the tube. If citrated blood is obtained with a syringe, aspiration should be carefully performed, since high vacuum can lead to activation of the coagulation system. Alternatively, samples for routine tests may be collected via an indwelling intravenous catheter (Maeckelbergh and Acierno, 2008), which, in dogs, results in acceptable agreement with values from blood samples collected via direct venipuncture. Use of samples collected via an intravenous catheter to monitor routine coagulation tests in critically ill patients eliminates additional venous trauma and discomfort and may also reduce the volume of blood getting lost during sample collection (Maeckelbergh and Acierno, 2008).

To guarantee intensive mixing of the collected blood with the anticoagulant (sodium citrate), tubes (or syringes) should be swayed carefully already while the blood is being collected. After the blood collection is finished, sample container must be carefully swayed and revolved several times. Haemolysis



should be avoided (Lippi et al., 2012). In vitro addition of haemolysate significantly interferes with coagulation times such as APTT and (thrombin time) TT in canine plasma (Moreno and Ginel, 1999), although illustrations indicate only limited absolute effects and no clear concentration-dependency. In addition, studies on human sample material showed only negligible differences of prothrombin time (PT) and APTT between 50 paired haemolysed and recollected non-haemolysed samples and between in vitro haemolysed samples and non-haemolysed controls (Laga et al., 2006), possibly making it unnecessary to reject haemolysed samples for routine tests.

### **Citrated plasma preparation**

Centrifugation of the sample and subsequent immediate removal of the (platelet-poor) citrated plasma supernatant using a pipette should be performed as quickly as possible, at the latest within 2 hours after blood collection. Immediately before centrifugation for 10 minutes at 1,500–2,000 x g, the sample should be checked for possible clots and coagulated samples must be discarded.

### **Storage stability, sample transport**

Only minimal, mostly non-significant changes in routine coagulation tests (PT, APTT), vast majority of the coagulation factors, antithrombin activity, and D-dimer concentration occur after storage of normal citrated plasma samples at room or refrigerator temperature for up to 48 hours (Furlanello et al., 2006). After 24 hours, the only significant difference was a reduction of fibrinogen concentration in samples stored at 24 °C (mean values: initially 1.95 g/l, after 24 hours: 1.87 g/l). These results contrast another storage study, where mean plasma fibrinogen concentration in normal canine plasma declined from initial mean values of 3.2 g/l to 2.9 g/l and 2.3 g/l, respectively, after storage for 8 or 24 hours at 8 °C (Piccione et al., 2010). Canine von Willebrand factor (vWf) in plasma stored at either 4°C or 22°C (and even in blood when stored at 22°C) is also relatively stable for up to 48 hours (Johnstone et al.,

1991). Based on these findings, shipment via overnight express of citrated plasma to specialised laboratories seems possible and does not require cooling or freezing. However, the storage stability of coagulation proteins in citrated plasma has to be confirmed for pathological and feline sample material. In citrated whole blood from 40 dogs showing various diseases stored at room temperature (comment: procedure is not recommended, plasma should be separated as soon as possible, see above), median APTT decreased from 14.2 s (baseline) to 12.6 and 12.0 sec after a storage time of 24 or 48 hours, respectively, possibly indicating pre-activation of the contact phase (Maunder et al., 2012). This artificial shortening of APTT can mask mild factor deficiencies.

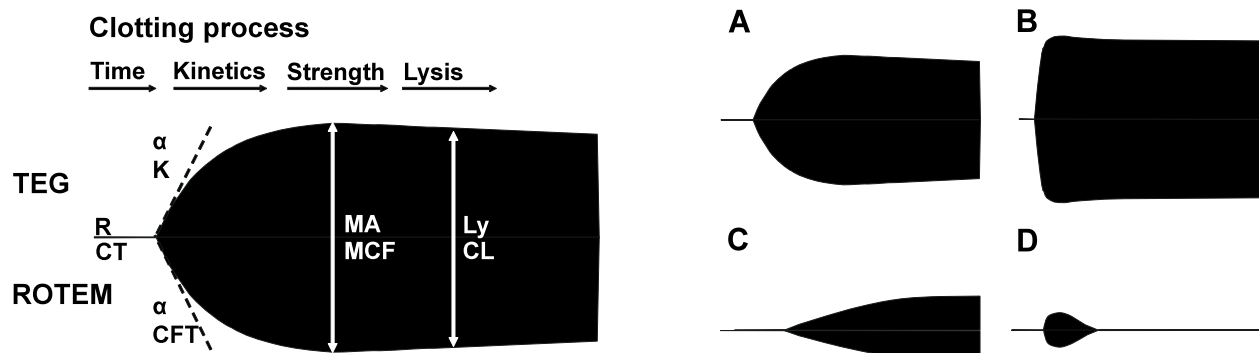
Platelet function is less stable and it is generally recommended to perform analyses (see below) within 3–4 hours after sample collection.

## **GLOBAL TESTING**

Global tests assess the overall haemostatic function of a blood sample including the relationship between platelets and the coagulation system. These include simple tests such as the whole blood clotting time or the activated clotting time (ACT) (Bateman et al., 1999) and automatic viscoelastic point-of-care haemostatic assays, which undergo a revival (Kol and Borjesson, 2010). These methods are easily to perform and can be used as bedside tests. Generally, these methods have limited sensitivity with respect to the detection of changes of individual haemostatic components.

### **Thrombelastography and thrombelastometry**

The two currently leading point-of-care haemostatic test systems are the thrombelastography (TEG®) and rotation thrombelastometry (ROTEM®). Blood is collected in a cup heated at 37 °C. Either native blood or recalcified citrated blood can serve as sample material, in each case without or with addition of activating agents (e.g., tissue factor, kaolin). A pin is suspended within the cup connected



**Figure 3.** Schematic TEG/ROTEM traces indicating: (a) the commonly reported variables (1) reaction time (R)/clotting time (CT), (2) clot formation time (K/CFT), (3) alpha angle ( $\alpha$ ), (4) maximum amplitude (MA)/maximum clot firmness (MCF), and (5) lysis (Ly)/clot lysis (CL) as well as (b) different characteristic patterns: (A) normal, (B) hypercoagulability, (C) hypocoagulability (e.g. thrombocytopenia/thrombocytopathia), (D) primary hyperfibrinolysis.

to a detector system (a torsion wire in the TEG and an optical detector in the ROTEM). The cup and pin are oscillated relative to each other through an angle of  $4^\circ 45'$ . The movement is initiated from either the cup (TEG) or the pin (ROTEM). When fibrin forms between the cup and pin, the transmitted rotation from the cup to pin (TEG) or the impedance of the rotation of the pin (ROTEM) are detected at the pin and registered as a graph. The different parts of the graph reflect different stages of the haemostatic process (clotting time, kinetics of fibrin formation, strength of the clot, and fibrinolysis). The nomenclature is slightly different between TEG and ROTEM (Figure 3a).

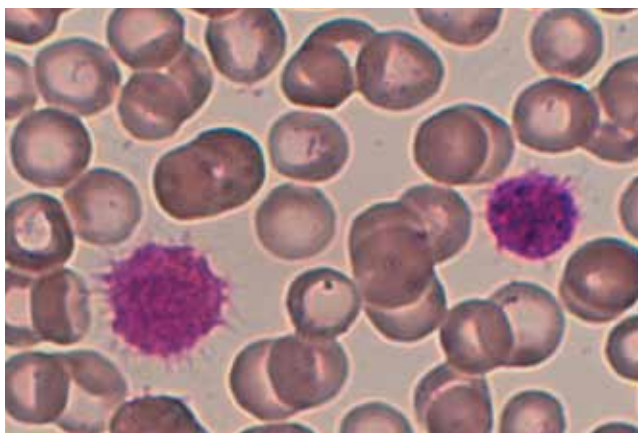
The method assesses the viscoelastic properties of clotting whole blood, reflecting interactions between cellular (platelets, erythrocytes, leukocytes) and plasmatic elements, and also of fibrinolysis under low shear conditions (Kol and Borjesson, 2010). Different haemostatic disorders are indicated by characteristic tracings (Figure 3b), whereas the automated numeric analysis of the graph is less important. Thereby, viscoelastic tests give an impression of the total haemostatic potential of a sample. In contrast to clotting tests, the method informs about the strength/stability of the clot and a possible lysis. It seems especially suited to detect hypercoagulable states (Wiinberg et al., 2008).

## BASIC EXAMINATION PROFILE

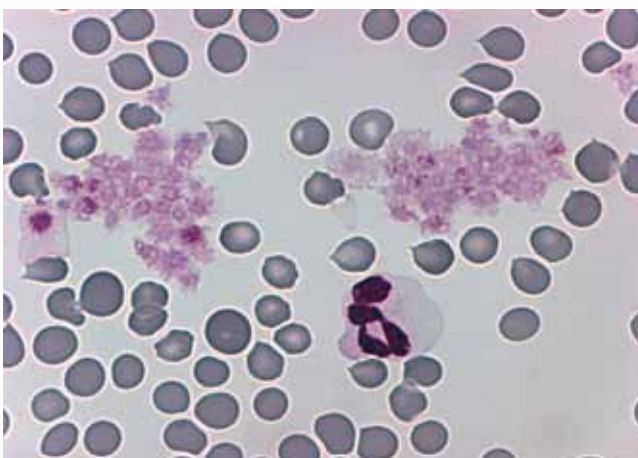
Basic examination profile for haemostasis usually includes platelet count, PT (syn.: thromboplastin time) and APTT. It may be expanded by thrombin (clotting) time and/or fibrinogen concentration measurements when PT and APTT are prolonged, to further differentiate whether the final stage of blood coagulation (thrombin-fibrinogen-interaction, fibrin formation) is affected. Additional measurement of capillary bleeding time is indicated in cases where platelet dysfunction or von Willebrand's disease are suspected.

## Platelet count

The platelet count can be estimated in a peripheral blood smear which, for example, can be used to verify thrombocytopenia measured by an automated blood cell counter. In addition, microscopic evaluation provides valuable information on platelet morphology (size, activation status, aggregates). Each platelet in an oil immersion lens field (1000 x total magnification) represents approximately  $15 \times 10^9$  platelets/l (McConnell, 2000). Thus, for example, a median of more than 10 platelets/oil immersion field represents an adequate platelet count in dogs. Large platelets (shift platelets or megathrombocytes) may be seen in conditions in which there is excessive platelet destruction and a regenerative platelet response with the exception of primary



**Figure 4.** Two large platelets (giant platelets) in the blood picture of a Cavalier King Charles spaniel suffering from macrothrombocytosis (Pappenheim stain, 2500x)



**Figure 5.** Platelet aggregates in the blood film of a cat indicated that automated platelet count (23,000/ $\mu$ l) was incorrect. Visual count revealed a true platelet count of 270,000/ $\mu$ l (Pappenheim stain, 1000x).



**Figure 6.** Test system for visual platelet counting: A capillary tube with 20  $\mu$ l of EDTA blood is placed into a sample tube containing a haemolytic and dilution medium (Thrombo Plus™ system). The sample applicator stick (demonstrated on the left) which is attached to the lid of the sample tube can be used to load the haemocytometer by capillary action.

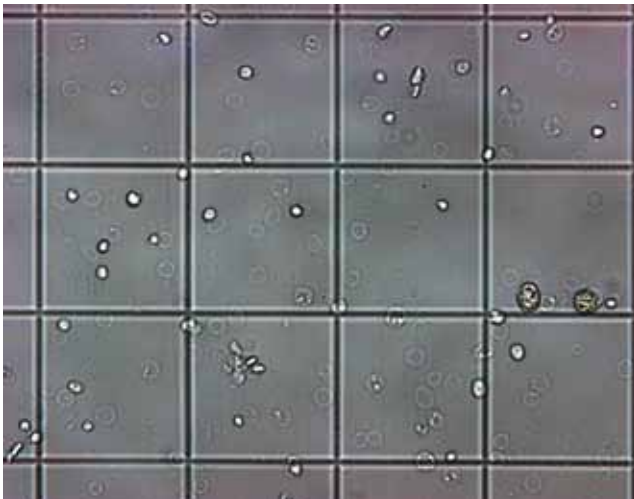
immune-mediated thrombocytopenia (Dircks et al., 2009). They may also be seen in infiltrative diseases of the bone marrow and as a frequent breed variant in Cavalier King Charles Spaniels (Cowan et al., 2004) (Figure 4). Small platelets (microthrombocytosis) may be seen in iron deficiency anaemia.

Automated cell counters often provide inaccurate platelet counts in cats because of the small size of red blood cells in cats and the tendency of feline platelets to form aggregates (Tasker et al., 2001) (Figure 5). Spurious thrombocytopenia occurs in Cavalier King Charles Spaniels, caused by the high frequency of large platelets. These dogs have usually a normal haemostatic function, which seems to be best reflected by plateletcrit determined by the IDEXX VetAutoread Hematology Analyzer (IDEXX Laboratories, Westbrook, USA) performing quantitative buffy coat analysis or by the Advia 2120 Haematology system (Siemens Healthcare Diagnostics, Eschborn, Germany) (Tvedten et al., 2008, Tvedten et al., 2012). False counting can also occur if small or large platelets are not detected by the machine because of inappropriate machine settings or calibration. Commercial kits (e.g., Thrombo-Plus, Sarstedt, Nümbrecht, Germany) simplify visual platelet counting using a counting chamber (Figures 6, 7). Healthy dogs have platelet counts of 150–500  $\times 10^9/l$  and healthy cats of 180–550  $\times 10^9/l$ . Breed may have an influence (Lawrence et al., 2013) which is currently inappropriately considered. Usually platelet number must decrease below 60  $\times 10^9/l$  to cause a significantly increased bleeding risk.

#### Further tests to verify cause of thrombocytopenias

Thrombocytopenia is one of the most important causes of haemorrhagic diathesis. Possible pathomechanisms include reduced production due to bone marrow diseases (e.g., hypoplasia, leukaemia, myelodysplasia) or increased turnover (loss, distribution abnormalities [splenomegaly, septicemia] or consumption [immune-mediated, disseminated intravascular coagulation/DIC, vasculitis]). Various laboratory tests help to verify the underlying



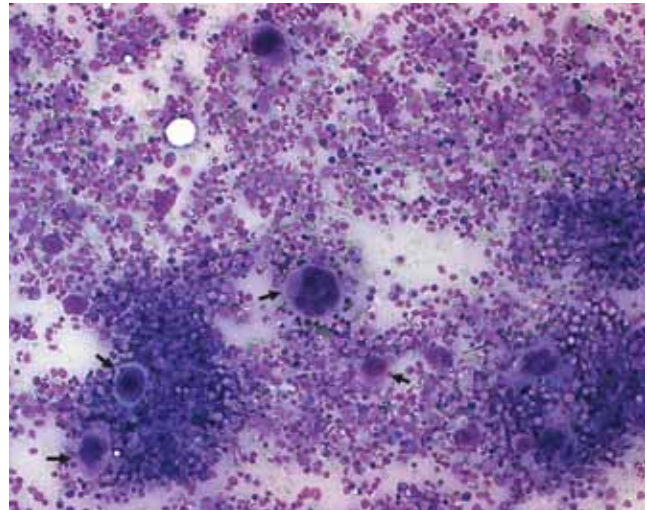


**Figure 7.** In the counting chamber, platelets are detected as small dots with a light centre and must be differentiated from the larger white blood cells (in the middle of the right hand side, two white blood cells can be seen; cell shadows in the background represent lysed red blood cells).

ing pathomechanism and disease. Bone marrow examination does not only assess thrombopoietic activity (reference values in adult female dogs:  $10.4 \pm 2.67$  platelet precursors per low power field and in male dogs:  $7.84 \pm 2.04$ ,  $P = 0.0008$ ) (Mischke et al., 2002), but may also offer further clues on the underlying disease (Figure 8). Increased mean platelet volume can indicate increased thrombopoietic activity, but may be low in primary immune-mediated thrombocytopenia despite high thrombopoietic activity (Dircks et al., 2009). Reticulated platelets are an alternative parameter of thrombopoietic activity. Flow cytometric detection of platelet-associated antibodies is helpful, but this test does not differentiate between primary and the more frequently occurring secondary immune-mediated thrombocytopenias (Dircks et al., 2009).

### Capillary bleeding time

The (capillary) bleeding time is the time until a standardised capillary bleeding ceases and a useful in vivo screening test for the function of primary haemostasis. It is indicated in patients with suspected platelet function disorder or von Willebrand's disease, i.e. in patients with haemorrhagic diathesis with



**Figure 8.** Significantly increased number of platelet precursors (partly indicated by arrows) in the bone marrow aspirate of a dog with severe thrombocytopenia ( $20,000/\mu\text{l}$ ) indicates an underlying immune-mediated pathomechanism (Pappenheim stain, 100x).

normal platelet count and clotting tests. Furthermore, it is a useful pre-surgical screening test for ruling out defective primary haemostasis in an animal with no contemporary clinical evidence of bleeding.

Capillary bleeding time in dogs is preferably measured in the non-anaesthetised dog in lateral position (Nolte et al., 1997). This method is, according to the experience of the author, superior to the widely used buccal mucosal bleeding time (BMBT) (Jergens et al., 1987) with regard to feasibility, possibility of standardisation, and safety purposes (i.e., possibility to stop bleeding with a bandage in case of a severe haemostatic disorder).

The skin of the lateral side of a front toe is shaved and a hyperaemic agent is applied for 1 minute to the shaved area and wiped off (Figure 9). A sphygmomanometer cuff, which was earlier placed above the antebrachium, is pumped up to apply a pressure of 60 mm Hg. This is performed to increase and standardise blood flow in the puncture area. One minute after applying the pressure of 60 mm Hg, the skin is punctured twice 5 mm apart, with a sterile manual or semi-automatic blood lancet, close to the edge of the horny skin of the pad (Figure 10). The blood is carefully dabbed off every 15 seconds with a swab until the bleeding stops (i.e., until there are no traces of blood

noticeable on the swab). The average time from the two punctures is calculated as capillary bleeding time.

The reference values of this method in the dog are 1–2.5 minutes. The reference ranges for the BMBT in dogs are 1.7–4.2 minutes (Jergens et al., 1987) and in cats 1.0–2.4 minutes (Parker et al., 1988). Result depends significantly on the device (Aumann et al., 2013). Prolonged capillary bleeding times can be caused by severe thrombocytopenia, platelet function disorders or von Willebrand's disease. In patients with normal platelet count, capillary bleeding time serves as a screening test of platelet function disorders and von Willebrand's disease. Severe von Willebrand's disease can be associated



**Figure 9.** Capillary bleeding time: A non-anaesthetised dog is held in lateral recumbency and a hyperaemic agent is applied to the shaved lateral side of a front toe.



**Figure 10.** A blood lancet is used to make two punctures close to the edge of the keratinized skin of the footpad. The bleeding time is detected by carefully dabbing the blood from these two sites every 15 sec with a swab until the bleeding stops.

with extremely prolonged haemorrhage from puncture sites and may require application of a bandage.

### **Blood coagulation tests (global tests, group tests)**

Coagulation tests measure the time taken for citrated platelet-poor plasma to clot when coagulation is initiated by the addition of calcium and activating agents. Blood coagulation tests are usually performed with coagulometric methods, but can also be performed with chromogenic assays. Coagulation times can be measured by manual techniques (tilt tube, hook technique) with visual detection of the time point of clot formation, but these techniques require considerable practice to obtain reliable results. Preferably, semi-automated or automated coagulometers are used, which are based on different electro-mechanical (e.g., Schnitger and Gross coagulometer based on the hook technique, ball coagulometer) or photo-optical techniques which detect a change in the intensity of light transmission when clot formation occurs. In addition, point-of-care analysers with test cartridges for PT and APTT are available for animals (e.g., VetScan VSpro Coagulation Analyzer; Abaxis Europe, Darmstadt, Germany). A recent study demonstrated an acceptable correlation with conventional PT and APTT assays (Dixon-Jimenez et al., 2013).

In order to test the plasmatic coagulation system rationally, the screening tests PT and APTT are widely used. These tests evaluate the whole plasma coagulation system with the exception of factor-XIII-dependent fibrin cross-linking (Figure 11). Each of these “group tests” records several coagulation factors of the extrinsic and common or intrinsic and common pathway, respectively. When PT and aPTT are prolonged, the complementary measurement of the thrombin time, the third group test, is indicated. Measurements of these parameters in canine and feline plasma are typically performed using tests optimised for the determination of human plasma, but species specific features suggest test modifications and/or must be considered when interpreting test results.

### Prothrombin time

Prothrombin time imitates the coagulation activation via the extrinsic pathway and measures the coagulation factors of the extrinsic and common pathway. Clotting is initiated by adding calcium and tissue thromboplastin, which provides tissue factor and substitutes the negatively charged phospholipid surface provided by platelets *in vivo*.

Prothrombin time measured in canine or feline plasma following the manufacturers' test instructions ("PT standard test") is significantly shorter than in human beings and, consequently, insensitive for indicating decreased coagulation factor activity. In specialised laboratories, the use of an optimised test (e.g., 100 µl 1:20 diluted sample, 100 µl fibrinogen solution [to guarantee an adequate fibrin clot formation], incubation for 2 minutes, 100 µl Ca-thromboplastin reagent) is preferable to the standard test for use in dogs and cats (Mischke et al., 1996; Mischke and Nolte, 1997). Apart from the modified test, commercial PT test modifications such as Hepato Quick or Normotest are suitable screening tests of the extrinsic system in dogs and cats (Mischke, 2002).

In general, reagents based on animal-derived

thromboplastins seem better activators of animal samples than synthetic/recombinant human thromboplastins. High inter-batch stability indicates that the preparation of lot-specific references is not required. Calculation of percentage activity values or PT ratios may minimise differences between different lots of one defined reagent, if an adequate and consistent standard material is available. However, even after converting the result into a prothrombin ratio or in percent of normal activity (compared to a reference curve prepared with a canine or feline pooled plasma), it is impossible to get a reagent-independent result (Mischke, 2010).

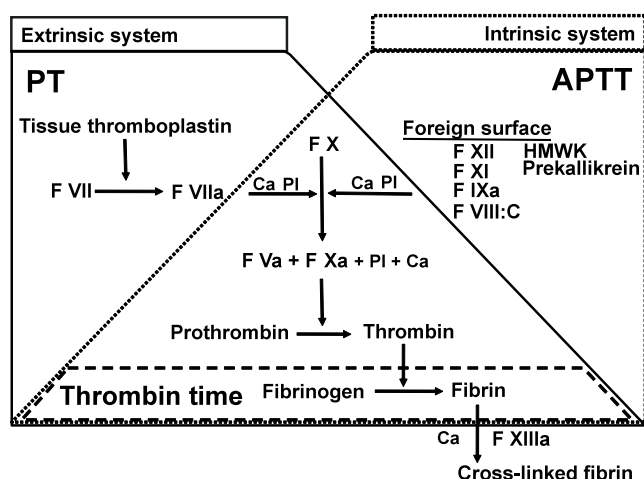
Prolongation of the PT standard test indicates a severe deficiency in clotting factors, because factors must decrease to less than 30% of normal to cause prolongation of PT, whereas the optimised test can also detect milder factor deficiencies. The PT is frequently prolonged in acquired vitamin K deficiency, liver disease, specific factor deficiencies and DIC. Factor VII deficiency causes prolongation of the PT with no change in APTT and thrombin time. Due to the short half-life of factor VII, the PT is selectively prolonged in the early stages of acquired vitamin K deficiency.

### Activated partial thromboplastin time

APTT imitates coagulation activation via the contact phase and identifies coagulation factor abnormalities of the intrinsic (high-molecular weight kininogen, prekallikrein, factors XII, XI, IX and VIII) and common pathway (factors X, V, II, and fibrinogen).

Clotting is initiated by adding a surface activator (such as ellagic acid, silica or kaolin), phospholipid (which substitutes the negatively charged phospholipid surface provided by platelets *in vivo*), and excess calcium. The reagents used in the APTT vary greatly in composition and therefore in sensitivity and specificity. Dilution of the plasma does not increase the sensitivity of this test when using electro-optical detection of fibrin formation (Johnstone, 1984).

With sensitive APTT reagents (e.g. Hemos IL SynthAFAX, Instrumentation Laboratory, Bedford,



**Figure 11.** Classical presentation of the haemostasis system indicating areas examined by the three group tests prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT).

Abbreviations: F – (coagulation) factor; HMWK – high molecular weight kininogen; PL – phospholipid; Ca – Calcium ions.



USA), APTT test optimised for human plasma can even detect mild individual factor deficiencies in canine and feline plasma (Mischke, 2000). Haemophiliacs have usually prolonged APTTs, dogs with haemophilia A of approx. 1.5 to 2.5-times normal. With less sensitive reagents even significant factor deficiencies to values  $< 30\%$  may result in false negative (normal) results. Haemophilic carriers with residual activities of usually 40–60 % factor VIII or IX can not be detected with sufficient certainty. Optimal APTT ratio for heparin treatment (i.e., corresponding to 0.3 to 0.7 I.U./ml) must be assessed by APTT-reagent and heparin-preparation specific calibration.

#### ***Thrombin time (thrombin clotting time)***

Thrombin time measurements are indicated in cases of combined prolongation of PT and APTT to assess, whether the final fibrin generation process is involved in the haemostatic disorder. In addition, thrombin time is used to control heparin therapy.

Thrombin time measures the clotting time of citrated plasma after the addition of exogenous thrombin. The result depends on the availability of an adequate concentration of fibrinogen and possible inhibitors of thrombin-fibrinogen-interaction (heparin) and fibrin polymerisation (fibrin[ogen] degradation products, abnormal serum proteins).

Commercial test kits are suitable to measure thrombin time in dogs and cats. Thrombin time in dogs and cats gives very similar results to humans. The thrombin time is prolonged, if the fibrinogen concentration is less than 0.7 g/l. In samples containing a normal fibrinogen concentration, fibrinogen degradation products cause prolongation at concentrations of  $\geq 0.1$  mg/ml (Mischke and Wolling, 2000). High thrombin concentrations are required to monitor animals receiving therapeutic doses of unfractionated heparin (Mischke and Jacobs, 2001).

#### **Reference values**

The use of animal species-, method-, and reagent-specific reference values is important for the

interpretation of results of group tests and also of other haemostasis test procedures. These reference values are best established in the laboratory itself and should be based on a sufficient number of healthy animals of the respective species (at least 40).

#### **Interpretation of test results of the basic programme**

Combined interpretation of results from platelet count and group tests and, if necessary, capillary bleeding time, often allows a tentative diagnosis to be made, which partially needs confirmation or further differentiation by further tests, especially individual factor analyses (Tab. 1).

#### **FURTHER AND SPECIFIC TESTS**

In individual cases, additional specific tests are necessary to confirm diagnosis or to accompany therapy.

#### **Blood clotting factors**

Determination of plasma activities of individual clotting factors is important for the diagnosis and monitoring of haemostatic disorders, particularly hereditary individual clotting factor deficiencies such as haemophilia A and B. Factors II, V, VII, and X are measured with a modified PT test, factors VIII, IX, XI, and XII with a modified APTT. The tests are performed with a diluted sample and addition of a deficient plasma that is deficient in a single individual factor. Therefore, the coagulation time of the test depends on the residual activity of the requested coagulation factor.

Coagulometric tests that incorporate human deficient plasma are typically used for the determination of clotting factor activities in dogs and cats. Due to the fact that particularly factor V and VIII:C activities in canine and feline plasma are several times as high as in human plasma, accurate measurement of coagulation factor activities in canine or feline plasma, using human deficient plasma, requires higher sample dilution (i.e.,  $1:>20$ ) than typically used for



human plasma (Mischke, 2001).

Differences in activities between human and canine or feline plasma and non-parallelism of the standard curves emphasize the necessity of use of species-specific standard curves for accurate determination of clotting factor activity. Standard curves are prepared with different dilutions of species-specific pooled plasma whose activity is usually defined as 100 %. Factor activities of 30 % are usually sufficient for a physiological haemostatic function. Haemophilic patients have usually < 10 % of the respective coagulation factor.

The fibrinogen concentration is usually determined in an extended coagulation profile and also with regard to its role as an acute phase reactant, mostly using the functional clotting method according to Clauss (Ameri et al., 2011). Commercially available standard curves for human fibrinogen or human standard material can be used for calibration, because calibration curves prepared with canine or feline specific fibrinogen standards show very similar results. The fibrinogen concentration ranges from 1.0 to 3.0 g/l in normal dogs and cats. Hyperfibrinolysis occurs frequently including animals with DIC. Hypofibrinogenaemia occurs primarily because of increased fibrinolysis in DIC with dominant hyperfibrinolysis.

### **Inhibitors**

The most important inhibitors of the blood clotting system are antithrombin (AT) and protein C. Antithrombin measurement is particularly necessary in cases of DIC receiving high dose heparin treatment to guarantee an adequate AT level. Heparin mainly functions by enhancing the inhibition of thrombin by AT and, thereby, increases AT consumption. Assessment of AT activity is also useful in animals suspected of having a hypercoagulable state. Common assays for AT activity are based on synthetic factor Xa- or thrombin-dependent chromogenic substrates and well suited for measurement of antithrombin activity in animals (Mandell et al., 1991).

Canine and feline pool plasmas are ideal standards. Subnormal AT levels (< 75 %) are associated with decreased heparin effectiveness. AT concentrations decrease in DIC. In one study, 85% of dogs (35 of 41) with confirmed DIC had decreased AT levels (Feldman et al., 1981). AT also decreases in hepatic disease owing to decreased synthesis. AT is similar in size to albumin and is lost in protein-losing nephropathies and protein-losing enteropathies.

### **Activation markers**

Plasma markers which are useful to detect an activation of the clotting system in small animals (e.g. thrombosis, DIC) include D-dimer, soluble fibrin and thrombin-antithrombin complex concentrations. Because antibodies against human D-dimers cross-react with canine and feline D-dimers, human tests are suitable. Immunoturbidimetric methods are useful reference methods (Boutet et al., 2009). Point of care cartridge tests are available and partly evaluated for canine samples (e.g., NycoCard D-dimer test and Nyco-Card READER II, Axis-Shield Point-of-Care Division, Oslo, Norway) (Dewhurst et al., 2008).

Healthy dogs have D-dimer concentrations of less than 0.25 µg/ml. Because generation of D-dimers requires actions of thrombin and plasmin, increased D-dimers are markers of intravascular clotting. A negative result more or less rules out thrombosis, but a positive result is relatively unspecific (Nelson and Andreasen, 2003). Many DIC scores include increased D-dimer levels.

### **Von Willebrand's disease**

Von Willebrand diagnostics is indicated especially in animals with prolonged bleeding time that cannot be explained elsewhere (especially by thrombocytopenia). Furthermore, measurements of vWF is indicated in cases with suspected haemorrhagic disorder, which cannot be captured by measurement results of platelet count and clotting tests. In most cases, measurements of vWF concentration is adequate. This can be performed with human test kits based on latex agglutination or ELISA test principles due to cross-reactivity

of the antibodies. Specific assays (collagen binding assays, electrophoretic multimeric analysis) detect a selective loss of the functionally active large multimers to characterize a Type II von Willebrand's disease (Burgess and Woods, 2008).

Canine pooled plasma serves as the standard. VWF has a wide reference range (50–180 %). It is an acute phase reactant and may be increased in conditions such as strenuous exercise, age, azotaemia, liver disease, parturition and after application of the vasopressin analogue, 1-desamino-8-D-arginine vasopressin (DDAVP).

### Platelet function analysis

In cases of prolonged capillary bleeding time despite normal platelet count and exclusion of von Willebrand's disease, functional platelet tests can provide further information with respect to the underlying cause. Further indications exist in patients suffering from primary diseases frequently associated with platelet functional disorders (e.g., liver disease) or for monitoring and evaluation of anti-platelet drug treatment. Finally, global tests of primary haemostasis such as capillary bleeding time and automated platelet function analysis are indicated for the estimation of the overall function of primary haemostasis in thrombocytopenic dogs requiring surgeries. To characterise functional platelet disorders, a wide variety of platelet function tests is available which, unfortunately, often lack standardisation. Apart from the "global tests" cited above these include, for example, platelet aggregation, flow cytometric analyses, thromboelastography (esp. "TEG platelet mapping" which is performed with special reagents) (Brainard et al., 2010) and thrombelastometry as well as mean platelet component.

Whereas primary functional abnormalities of platelets (e.g., Glanzmann's thrombasthenia; signalling defects, storage pool deficiency, and Chediak-Higashi syndrome) are extremely rare in animals (Boudreaux, 2008) and in humans, acquired platelet dysfunctions are more frequent and occur associated with many diseases and disorders (e.g. hepatopathy,

monoclonal gammopathy) and after application of anti-platelet agents (aspirin, clopidogrel) and other drugs (NSAIDs, dextran, antibiotics).

### Platelet function analyser (PFA 100)

The platelet function analyser PFA-100 (Siemens Healthcare Diagnostics, Germany) has been evaluated and is suitable for use in dogs (Callan and Giger, 2001; Mischke and Keidel, 2003). The instrument comprises a microprocessor-controlled instrument and disposable test cartridges containing a biologically active membrane. The instrument aspirates a blood volume under constant vacuum from a sample reservoir in the test cartridge through a capillary and a microscopic aperture cut into the membrane at the end of the capillary (Figure 12). The membrane is coated with collagen and adenosine diphosphate (collagen/ADP cartridge) or collagen and epinephrine (adrenaline) (collagen/epinephrine cartridge). The presence of these biochemical stimuli, and the high shear rates developed under standardized flow conditions, result in platelet attachment, activation, aggregation, and slow formation of a stable platelet plug at the aperture. The "closure time" is reported by the analyser.

The analysis should be performed between 30 min and 2 h after collection of the citrate buffer-anticoagulated blood which is the standard sample material (Mischke and Keidel, 2002). It is advisable to use only one position, e.g. position A, of the two alternative measuring positions of the analyser, because spontaneous sedimentation can lead to artificial results in the second cartridge inserted into position B.

Reference values are as follows: collagen/ADP cartridge: 52–86 s (Callan and Giger, 2001), 53–98 s (Mischke and Keidel, 2003); collagen/epinephrine cartridge: 97–225 s (Callan and Giger, 2001), 92–>300 s (Mischke and Keidel, 2003).

The method can be regarded as a global screening test of primary haemostasis. Apart from platelet function, the result is significantly influenced by platelet count and haematocrit (Callan and Giger, 2001; Mischke and Keidel, 2003). Unfortunately,

even slightly subnormal haematocrit values are associated with a prolongation of the closure time which limits the clinical applicability of the analyser. Because measurements using the collagen/epinephrine cartridge exceed upper limit of the measuring range already in a small percentage of healthy dogs (Mischke and Keidel, 2003), this cartridge type is less suitable for tests of primary platelet function in dogs.

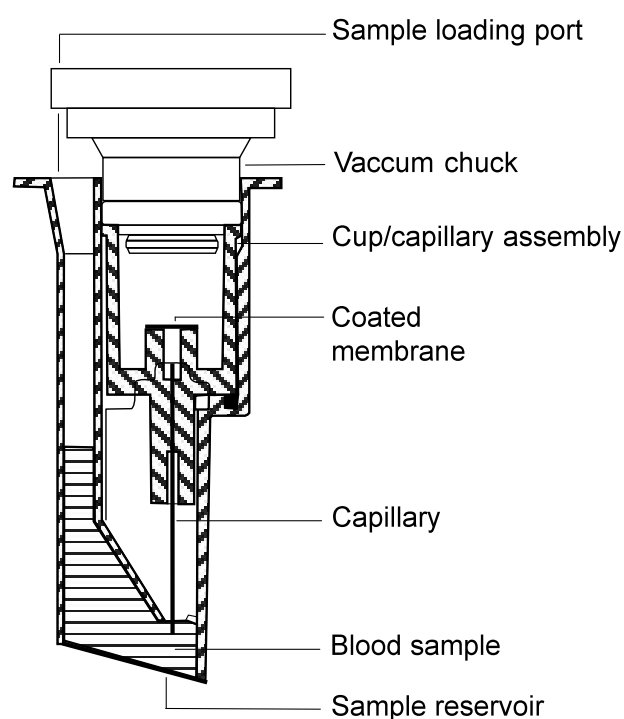
### Platelet aggregation

The measurement of platelet aggregation is one of the most important in vitro methods of assessing platelet function. There are two main measurement principles of in vitro platelet aggregation following the addition of agonists. The turbidimetric method measures the increase of light transmission in platelet-rich plasma during aggregation, while impedance aggregometry measures the increase in the electrical resistance in blood resulting from platelet accumulation (Dyszkiewicz-Korpanty et al., 2005). Although

the turbidimetric method has the advantage that the platelet count can be adjusted to a defined value, the preparation of platelet-rich plasma requires additional equipment and time, large sample volumes, and experienced personnel. This preparation procedure can also alter the quality of the sample through loss of large platelets with increased or decreased reactivity. Impedance aggregometry on whole blood can more effectively evaluate lipaemic samples. It may also better reflect in vivo platelet functionality, given that platelets can interact with other blood cells. The electrical probes of the impedance method create an artificial surface and therefore may better mimic in vivo platelet aggregation which typically occurs on injured or inflamed vascular surfaces.

The novel impedance aggregometer *Multiplate Analyser* (Roche Diagnostics GmbH GmbH, Mannheim, Germany) seems to be well suited for use in dogs (Kalbantner et al., 2010). The test can be performed as recommended by the manufacturer for human sample material (300 µl isotonic sodium chloride solution, 300 µL of hirudin-anticoagulated blood, 3 min incubation and stirring at 37°C, addition of 20 µL agonist solution) (Kalbantner et al., 2010). The impedance change caused by the adhesion and aggregation of platelets on the electrodes is continuously detected.

To achieve optimal sensitivity, minimum effective (threshold) concentrations of agonists should be used which reliably induce high maximum aggregation values in healthy animals. In a canine study using the Born method, the following threshold concentrations (final test concentrations) were established: 25 µmol/l adenosine diphosphate (ADP), 10 µg/ml collagen and 1 IU/ml thrombin (Mischke and Schulze, 2004). In an actual study using the Multiplate instrument, the following optimal agonist concentrations (sourced from Roche Diagnostics GmbH) were defined: 10 µmol/l ADP, 5 µg/ml collagen, and 1 mmol/l arachidonic acid (Kalbantner et al., 2010). Reference values should be established method- and laboratory-specific. Platelet aggregation is particularly suitable to detect decreased platelet



**Figure 12.** Schematic diagram of the cross-sectional view of the functional unit of the platelet function analyser PFA-100.

function. Because low agonist concentrations are associated with a wide range of results, it is almost impossible to detect hyperaggregability in individual patients. Platelet counts influence the result.

## CONFLICT OF INTEREST STATEMENT

The author of this paper does not have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper. ■

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