Haemostasis is the name of a group of processes initiated in the body in order to stop bleeding in case of tissue and/or blood vessel injuries. Haemostasis examinations should be started by fast tests that can be performed by side of the animals. These are not accurate but easy to perform and give good estimation. By doing these tests we can give a direction for more specific diagnosis of haemostasis disorders.

**Major groups of hemostasis disorders.**
1. Vasculopathy (decreased ability of vasoconstriction in case of blood vessel injury, the first step of the haemostasis process),
2. Thrombocytopathy (decreased ability of platelets to aggregate and adhere to the site of injury, and formation of the primary thrombocyte-thrombus, the second step of haemostasis) , - Thrombocytopenia (decreased amount of thrombocytes in the blood)
3. Coagulopathy (problems with the extrinsic-, intrinsic-, or common pathway of the coagulation cascade, which ends with the formation of a polymerised fibrin network, which keeps thrombocyte thrombi at the site of injury, the third and final step of haemostasis)

**Tests performed by side of the animals:**
- Signs of increased bleeding tendency: on the skin and mucous membranes: anemia, petechia, ecchymosis, suffusion; in the thoracic cavity: haemothorax; in the abdominal cavity: haemoperitoneum; in the gastrointestinal tract: haematemesis, melena
- Capillary resistance (human medicine)
- Bleeding time (buccal mucosal bleeding time test, BMBTT)
- Appearance of the first fibrin strand (clotting time)
- Appearance of the clot (clotting time on different surfaces)
- Clot retraction time

**Capillary resistance**
The test is usually used in the human medicine, also called Rumpel-Leed-test. It is performed by putting a ligature on the arm, above the elbow and checking the palmar side (because skin is thinner there) of the lower arm for petechie. After 3-5 min. of ligature 3 small petechie (small reddish dot, bleeding from capillaries under the skin) should appear normally. If capillary function is not proper, more petechia appear. Capillaries may become more fragile for example in case of vasculitis (viral, autoimmune, etc.), or other diseases affecting the wall of the blood vessels.

**Bleeding time, Buccal mucosal bleeding time (BT, BMBT):**
This is not the test for coagulopathies. But the test for thrombocytopenias, thrombocythopathies and vasopathies.
Use a sharp and sterile blade and make an at least 0.1-0.2 mm deep, 0.5 cm long incision on the skin of the inner part of the external ear or on the buccal mucosal surface (some animals will not tolerate this without mild
sedation, especially cats). Vipe the blood drop flowing UNDER the wound carefully with a cotton wool tissue, or paper towel in 20-30 sec. intervals. It is important to avoid touching the wound itself, because otherwise you may remove the small thrombocyte-thrombus from the wound, and therefore bleeding time will be longer. Measure the time from the appearance of the first drop of blood until the ceasing of bleeding. BT, BMBT is dependent on the thrombocytic function, the platelet count, and the capillary function. Normal BMBT: 3-5 min. There is no danger of clinical bleeding (appearance of petechia) until platelet count is above \( 50 \times 10^9/\text{l} \).

**Coagulation time (CT)**

These are the tests for coagulopathies!!!

It is always important to perform these tests from fresh, native (not containing any anticoagulants! ☺ ) whole blood samples, immediately after taking them! Samples should be taken by a proper way, preferably with only one precise venipuncture, so that we do not cause too much damage to the surrounding tissues. Otherwise we may cause increased tissue factor (factor III.) release from the damaged cells, which leads to the initiation of the coagulation cascade during sampling! It is advised to use the so called "two syringe method". In this case we do not use the first drops of blood for coagulation measurements, we change syringe after taking these first drops, and use the content of the second syringe for that purpose.

**Appearance of the first fibrin strand:**

Put some drops of the blood sample onto a glass slide and sink the tip of a needle into the blood and move the needle forward and back while carefully and slowly pulling the tip out of the blood sample. The first fibrin strand should appear within 1-2 min. You need to have good vision for the evaluation of this test, and a properly lighted room.

**Clotting time (CT) on watch glass:**

Put the fresh blood sample on watch glass that was previously treated with paraffin or wax (otherwise scratches of the glass would activate the coagulation cascade). Check the complete coagulation time (until the whole amount of blood becomes solid, like gelatine). Normal is 7-15 min.

**CT in plastic syringe**

Put the fresh blood sample into a plastic syringe (or leave some blood in it after sampling) and check the time of the complete coagulation. Normal is 10-12 min.

**CT in glass tube**

Pour some fresh blood into a glass test tube and check the time of the complete coagulation. Normal is 4-5 min.

**CT in ACT (activated clotting time) tube**

Put some fresh blood into a glass test tube that contains \( \text{SiO}_2 \) (commercially available tube, called ACT tube, usually with brown cap), put it in a thermostat (37\(^\circ\)C) and check the time of the complete coagulation. \( \text{SiO}_2 \) activates Factor XII (Hagemann-factor, contact factor). Activated Factor XII activates Factor IX and kallikreinogen, kininogen (fibronolytic pathway). Blood coagulation should be checked by slowly moving the tube every 15-20 sec. Normal is 3 min.

**Platelet (thrombocytic) count**
Platelet count examination is important especially, when the BT, BMBT is increased, or petechie are visible on the skin or mucous membranes. The platelet count should be measured from anticoagulated blood. For this purpose Na$_2$-, or K$_2$ EDTA should be used.

**Method 1**

Put 0.1 ml EDTA anticoagulated blood sample into 0.9 ml physiological saline solution, mix it, then let it to be sedimented for 2 hours. Then one drop of the upper layer should be dripped into Bürker chamber (haemocytometer). Wait until the drop is spread over the network, and count the number of platelets in 10 rectangles. The number should be multiplied by 10$^9$, and this is the number of platelets in 1 litre blood. The process can be made quicker if the sample in NaCl-solution is centrifuged on 1500/min. Thrombocyte counting in Bürker chamber is not accurate.

**Method 2**

Platelet count can be estimated by using a blood smear. Finding one platelet in one view by 1000x magnification means 20x10$^9$/l platelet count. This method is also very uncertain, however checking a blood smear for any purpose can be very important. The arrangement of thrombocytes may be variable on a smear, so we should check many views from the middle part and from the edges of the blood film. Finding big thrombocyte aggregates in the smear is suggestive of proper platelet function, and may explain low thrombocytic counts measured by automatic cell counters.

**Method 3**

Platelet count can be measured by using automatic cell counters. Particles of the blood between 5-30 fl volume are taken as platelets. Sometimes in regenerative processes of the bone marrow, when there are many young (big) platelets circulating i.e. in case of chronic blood loss, or physiologically in cats and in King Charles spaniels average thrombocytic volume can be so high, that these big platelets are taken as red blood cells by the counter. Sometimes small red blood cells are also taken as thrombocytes. Thrombocytic aggregates can be taken as white blood cells, so the platelet count is measured to be normal or low and the white blood cell count to be increased. Evaluation of the blood smear (examination the arrangement, morphology, etc. of thrombocytes) is a very important step of the diagnosis of thrombocytic disorders!

**General Platelet Count:** 200-800 x10$^9$/l

**Major causes of thrombocytopenia:**

1. decreased production of thrombocytes in the bone marrow
2. increased utilisation of thrombocytes: DIC (disseminated intravascular coagulopathy)
3. increased destruction of thrombocytes : autoimmune thrombocytopenia (AITP)
4. increased sequestration of thrombocytes: in case of (chronic) splenomegaly
5. increased loss of thrombocytes: subacute/chronic bleeding

**Clot retraction test**

If you leave the blood clot in a tube for some hours, it will become smaller, and serum will appear around the clot. The reason for this clot retraction is that platelets contain a contractile protein called thrombostenin, Normally the volume of serum released by the clot within one hour is approx. 25% of the whole volume of the initial clot.
Thrombocytic function can be estimated by performing this test. If the clot retraction is slower, or does not happen at all, we can suspect thrombocytopenia.

**Platelet aggregation test**

This test can be performed when we suspect thrombocytopenia, i.e. von Willebrand disease. In this case we can use aggregometers to estimate the aggregating ability of platelets correctly. There are several devices to evaluate the aggregation of the platelet. The methods are similar. We have to prepare a citrated blood sample and the upper layer should be used for this analysis. This is the platelet rich plasma. This fluid is slightly opaque. Then we put this sample into a cuvette and add some drugs which causes exaggerated aggregation, such as ADP, epinephrine etc. These drugs will induce platelet aggregation which will cause the clearing up of the fluid in the cuvette. The speed and the rate of the clearing can be analysed by a spectrophotometer. The device gives numerical values to this process. Other instruments are also available which are working by different methods.

**Thrombocytic morphology**

Thrombocytes have 1-2 µm diameter. Their centre is the granulomer and the edge is the hyalomer part (zone). Horses, sheep, cattles, have the smallest platelets (3-5 fl), dogs and swine have bigger (7-8fl), and cats have the biggest (10-15fl) thrombocytes.

Major causes of thrombocytopenies:
1. improper development of platelets, for example because of hereditary glucoprotein deficiencies, etc.
2. von Willebrand’s disease (see later)
3. uraemia, liver failure, myelo-, and/or lymphoproliferative diseases, NSAID (non-steroid anti-inflammatory drugs) treatment, etc.

In case of thrombocytopenia, thrombocytopenies and vasopathies besides normal coagulation, we cannot expect signs of a really severe bleeding disorder (suffusion, haematomata, haemothrorax, haemoperitoneum), because these are prevented by the formation of polymerised fibrin strands (fibrin thrombus) at the end of the coagulation cascade.

But coagulopathies, besides normal thrombocytic and blood vessel function and normal platelet count may lead to the aforementioned severe bleedings, and sometimes bleeding to death, because thrombocytic thrombi are not stable without a fibrin network, and in case of a big blood vessel injury, the power of blood flow can sweep away the thrombocyte-thrombus from the wound!
Major alterations of the basic tests in different haemostasis disorders:

<table>
<thead>
<tr>
<th></th>
<th>BT</th>
<th>CT</th>
<th>PC (platelet count)</th>
<th>Platelet morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulopathy</td>
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<td>←→</td>
<td>←→</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
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<td>altered or not</td>
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<tr>
<td>Thrombocytopathy</td>
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<td>←→</td>
<td>altered</td>
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<tr>
<td>Vasculopathy</td>
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<td>←→</td>
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</tbody>
</table>

Coagulopathies can be examined by using more specific “global “tests, so that we can evaluate, which group(s) of factors are not functioning properly. Citrated blood should be used (citrate prevents coagulation by binding calcium ions), which means, 3.8 % Na-citrate:blood = 1:9. (Prepared tubes are commercially available, usually blue cap)

**Prothrombin time (PT):**
This test must be performed within 1 hour after sampling. Blood samples should be mixed with 3.8% sodium citrate in 9:1 dilution (4.5 ml of blood and 0.5 ml of Na-citrate). Then centrifuge the samples in 3000 rpm for 10 min. and separate the (decalcinated) plasma from he sediment. The plasma must be kept on 37 °C.
The reagent (Simplastin) for PT evaluation contains rat uterous tissue homogenate as tissue thromboplastin (Factor III.), and CaCl₂. The reagent must be kept on 37 °C before using.
The evaluation can be performed by using coagulometer (see on the practical!) or in test-tube. 200 µl reagent should be mixed with 100 µl decalcinated (citrated!) plasma and the time of coagulation should be noted.
Normal PT: 10-15 sec.
This test gives information about the function of the extrinsic pathway, because the coagulation cascade is triggered by adding tissue factor (and calcium ion) to the decalcinated plasma sample.
Factors involved in PT are: VII., X., V., II., I., XIII.

**Activated partial thromboplastin time (APTT):**
For this test decalcinated plasma should be used similarly to PT. The reagent (Silimat) contains rabbit brain homogenate as PF₃ (platelet factor 3) and micronised silica as contact activator. 100 µl decalcinated plasma should be mixed with 100 µl reagent and the mixture kept on 37 °C, then 100 µl of 0.025 mmol/l CaCl₂ solution should be added and from this moment the time of coagulation should be noted.
Normal APTT: 20-30 sec..
This test gives information on the function of the intrinsic pathway, because the cascade is triggered by providing surface activation (imitating the effect of free collagen, which appears on the inner surface of the vessels in case of blood vessel injury), and adding platelet factor 3 (PF3) and Ca²⁺ for the activation of factor X. (remember the coagulation cascade!!).!
Factors involved in APTT are: XI., IX., VIII., X., V., II., I., XIII.
**Thrombin time (TT)**

By performing this test, decalcinated plasma should be simply mixed with a reagent containing thrombin only. In this case coagulation time depends on the concentration of fibrinogen and Factor XIII in the plasma. Obviously this test can also be used for the estimation of fibrinogen concentration (suspecting a normal factor XIII level in the blood).

<table>
<thead>
<tr>
<th></th>
<th>APTT</th>
<th>PT</th>
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</thead>
<tbody>
<tr>
<td>Intrinsic pathway problem</td>
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<td>↔</td>
</tr>
<tr>
<td>(haemophilia A-factor VIII. deficiency; haemophilia B-factor IX. deficiency; von Willebrand’s disease)</td>
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<td></td>
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<tr>
<td>Extrinsic pathway problem</td>
<td>↔</td>
<td>↑</td>
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<tr>
<td>(Factor VII deficiency, dicumarol toxicosis - first stage)</td>
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<td></td>
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<tr>
<td>Common pathway problem</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>(liver disease - dec. prod. of coag. factors, DIC, dicumarol toxicosis - second stage, Factor X. and/or V. and/or II. and/or I. and/or XIII. deficiency)</td>
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</tbody>
</table>

In the early stage of dicumarol (or warfarin) toxicosis only PT is increased, and later APTT is increased, too.

Dicumarol is a competitive antagonist of vitamin K. Vitamin K is responsible for the gamma carboxylation of Proconvertin (Factor VII), Christmas (Factor IX), Stuart-Prower (Factor X) and the Prothrombin (Factor II), as they are Ca$^{2+}$ dependent factors, so vitamin K deficiency causes the inability of these factors to bind calcium. Factor VII has the shortest half life, so this factor will be deficient first. Prothrombin Time is increased when there is factor VII deficiency, so this test will show the problem first.
Coagulation cascade

Collagene Tissue damage
(neg. charge, SiO₂)

XII III
XI
IX* VII*

VIII V X* II*

PF₃ TT I XIII

polimerised fibrin

Intrinsic Extrinsic
APTT PT

Signed factors (*) are the Vit-K dependent factors

Function of Vitamin K is to exaggerate the post-synthetic gamma carboxylation of those factors in the liver (vitamin K is the co-factor of the enzyme catalysing the attachment of a carboxyl group on the gamma C atom of the protein molecule after protein synthesis). This makes these factors to be able to bind Ca²⁺, and thus become functionally active.

In case of vitamin K deficiency of any origin (dicumarol toxicosis, etc.), improperly carboxylised prothrombin can be detected by using an ELISA (Enzyme Linked Immunosorbent Assay) test, called PIVKA II. (Proteins Induced by Vitamin K Absence)

**Fibrinolytic degradation products (FDP)**

Fibrinolytic pathway is responsible for keeping the clot formation within normal limits. Clot inhibitors (antithrombin III, alpha2-macroglobulin, alpha1-antitripsin, heparin – the latter increases the binding of antithrombin III to thrombin) are able to bind to thrombin and neutralise it.

After a complete coagulation, the clot is usually not needed any more, so it should be broken down by fibrinolytic enzymes. Free collagen fibres, (exposed at the site of blood vessel injury), kininogen and kallikrein are the activators of factor XII. (Hageman). XII.a (activated form of factor XII) further activates the extrinsic pathway, and is also able to form kallikrein from prekallikrein. Kallikrein activates the kininogen system, also, forming bradikinin (activated form of kininogen), which is a very potent mediator of pain. Kallikrein is the most important activator of plasminogen. Plasmin (activated form of plasminogen) is an endopeptidase, which can cleave fibrin strands into small pieces. Before the total degradation of polymerised fibrin strands, increased level of fibrinolysis-products, fibrin dimers and monomers (so called: “fibrin degradation products or proteins, FDP”) can be measured in the blood. FDP is not a proper name for these proteins, because, some of them are the
breakdown products of fibrinogen, also, not just fibrin. Therefore high FDP in the blood is not necessarily the sign of increased fibrinolysis following increased clot formation, because these products can also originate from fibrinogen. More accurate way to detect increased fibrinolysis is the examination of D-dimer level in the blood. This derivate is originated only from fibrin, and not fibrinogen. Both tests (FDP, and D-dimer) are based on latex agglutination method, and fresh citrated (decalcinated) plasma samples should be used for it. The reagent contains antibodies (produced in research animals) against FDPs or D-dimers attached to latex particles. The reagent should be mixed with fresh citrated plasma sample and macroscopic agglutination can be seen dark (black) surface if enough FDPs and/or D-dimers are present in the plasma. Performing each of these tests (FDP, or rather D-dimer) is very helpful in the early diagnosis of disseminated intravascular coagulopathy (DIC). DIC is a common acute disorder that requires accurate and quick laboratory diagnosis. It is usually a secondary problem, caused by primary diseases for example: septicaemia, pancreatitis, widespread burn injuries, or necrosis of big tumors, shock, polytraumatisation, etc. In case of DIC, microthrombus formation and fibrinolysis are present at many different places in the body simultaneously, because of severe tissue damage or necrosis (initiation of the extrinsic pathway, by the release of tissue factor), and blood vessel injury (initiation of the intrinsic pathway), so coagulation factors and platelets are consumed very quickly during this process. That is why it is also called “consumptional coagulopathy”. DIC can be a life threatening complication of the aforementioned diseases. Early diagnosis is very important in the management of this condition. First laboratory sign of DIC can be a positive FDP or D-dimer test.

**Laboratory diagnosis of DIC**

Coagulation time: ↑

Bleeding time: ↑

Platelet count: ↓

PT: ↑

APTT: ↑

TT: ↑

Fibrin degradation products (FDP), and D-dimer: ↑

Appearance of schysocytes and/or burr-cells in blood smear (damaged red blood cells, see later)

**Diagnosis of von Willebrand disease**

This disease is known in humans, and in case of dogs in Dobermann pinchers, often accompanied by hypothyroidism. In this disease the von Willebrand factor, or the complete Factor VIII is deficient. There are 3 main part of complete Factor VIII: von Willebrand factor – responsible for platelet adhesion and aggregation, VIIIc – is the antihaemophylic factor, and the Factor VIII related antigen – is the hapten that is the determinable part, and is bound strongly to von Willebrand factor. The dogs with this disease have increased BT, BMBT, decreased clot retraction ability and sometimes coagulation disorders, too. The specific diagnosis is based upon the detection of the lack of von Willebrand related antigen.
<table>
<thead>
<tr>
<th>Condition</th>
<th>BT, BMBT</th>
<th>Platelet count</th>
<th>APTI</th>
<th>PI</th>
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</thead>
<tbody>
<tr>
<td>Thrombocytopenia</td>
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<td>↔</td>
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<tr>
<td>Thrombocytopathy</td>
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<tr>
<td>Intrinsic pathway disorder</td>
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<td>(pl. haemophilia A, B)</td>
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<tr>
<td>Factor VII deficiency</td>
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<tr>
<td>(i.e. early stage of dicumarol-toxicosis)</td>
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<tr>
<td>Disorder of the Common pathway</td>
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<tr>
<td>(i.e. liver failure, Factor X deficiency, late stage of dicumarol-toxicosis etc.)</td>
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<tr>
<td>DIC</td>
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<tr>
<td>von Willebrand-disease</td>
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