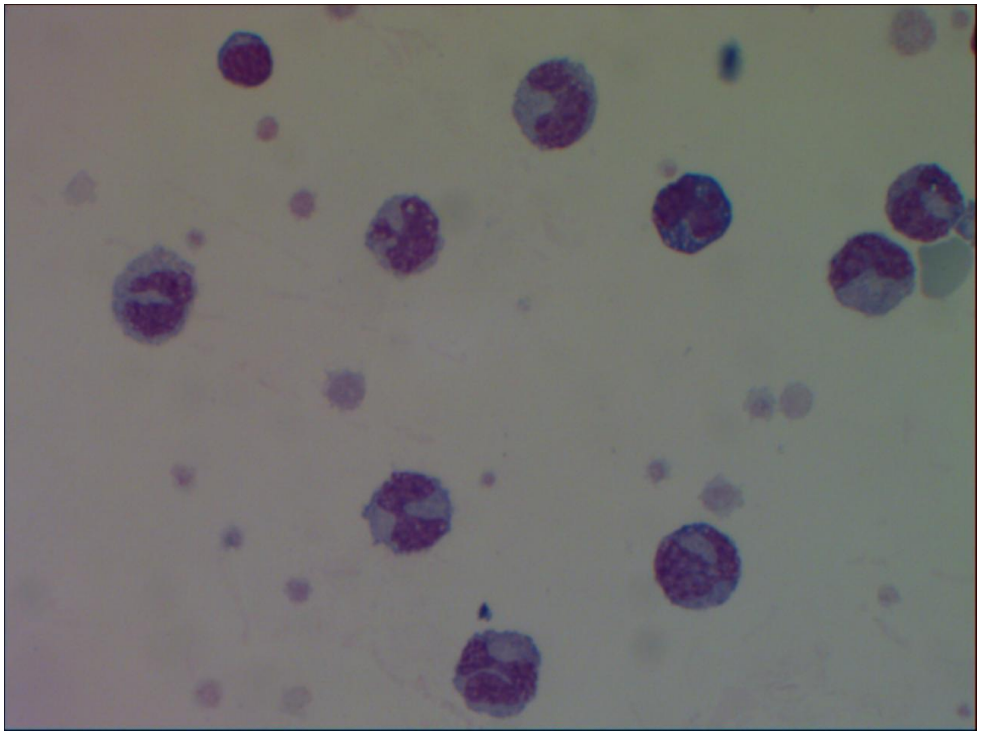

TISSUE FACTOR IN CANINE THROMBOCYTES

Tissue factor expression in thrombocytes of dogs suffering from idiopathic immune mediated haemolytic anemia

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General introduction

Dogs suffering from canine idiopathic immune mediated haemolytic anaemia (cIIMHA) are at great risk of dying particularly in the first two weeks after the diagnosis is made.*(1)* This high mortality risk may be associated with the development of thromboembolism (TE) *(2-6)*and/or disseminated intravascular coagulation (DIC)*(3)* and the resulting organ failure.

The exact mechanism of the development of TE and/or DIC in cIIMHA is undetermined. It might be caused by the presence of hypercoagulable platelets in these patients. Indeed, there are some studies which assume that dogs suffering from cIIMHA contain hypercoagulable platelets.*(2, 4, 7)* Those platelets may circulate in an activated state, which is determined by an increase of P-selectin expression on the platelet cell surface.*(7)* After platelet activation, P-selectin functions as a cell adhesion molecule and plays a role in thrombus formation by promoting platelet recruitment and platelet aggregation. Additionally, one veterinary study suggests that, in mice, monocyte-derived TF-bearing microvesicles bind to platelets by binding to platelet P-selectin and thereby donating TF to platelets.*(8)*

In dogs with cIIMHA there has been evidence of high intravascular tissue factor (TF) expression by blood cells.*(9)* In the context of the often existing monocytosis and the fact that monocytes are known TF sources, *Piek et al. 2011 (9)* assumed that maybe the monocytes are the source of TF expression in these patients. Indeed, previously some human research groups demonstrated that neutrophils and monocytes produce TF*(10-13)* and even donate it to platelets.*(14-16)* However, *Piek et al. 2011* could not confirm this in dogs with cIIMHA, because IL-8, a monocyte derived chemotaxin that normally triggers TF expression by monocytes, was significantly low in cIIMHA dogs compared to the other hypercoagulable patient-groups.*(9)* Therefore, in cIIMHA dogs you would have expected both high intravascular IL-8 and TF. Alternatively, they postulated that the platelets may contribute to the high intravascular TF expression in cIIMHA, because they found a statistically significant correlation between high TF production on one hand and decreases in both mean platelet volume (MPV) and mean platelet mass (MPM) on the other hand, which suggests platelet activation and therefore possibly TF expression by platelets.

Despite the scarce literature about veterinary research on canine platelet associated TF production, in human studies there has been done a lot of research. Some studies demonstrate that TF protein is bound on the platelet cell membrane *(17-24)*, whereas another study claims that activated platelets are also capable to release functionally active TF protein.*(19)* Moreover, not only activated platelets but also resting platelets are thought to contain TF protein (i.e. α-granules and in the open canicular system).*(21)* The origin of thrombocyte-associated-TF has been unknown for a long time, but recently a human study has shown that a subset of platelets inherits TF protein form their mother cell: the megakaryocyte.*(25)* Besides the demonstration of platelet derived TF protein, there is also some evidence that platelets contain TF mRNA*(20, 22, 23)* or TF pre-mRNA.*(26-29)* This is very interesting, because platelets are anucleated cells and therefore are biased to have no DNA inside. A possible explanation could be that platelets may have received TF mRNA from megakaryocytes that surely do contain a cell nucleus (even multiple cell nuclei). Indeed, this has been recently shown by *Brambilla et al. 2015*.*(25)*

Both the assumption from the study by *Piek et al. 2011* and the evidence obtained from human platelet studies suggests that canine platelets may also contain TF protein and/or TF mRNA. Therefore, the study objectives are **1)** to determine whether platelets in cIIMHA indeed contain TF mRNA and **2)** if they carry TF protein on their cell membrane. If so, then it may explain the increased risk of developing TE within the first two weeks of the disease. Additionally, we also wanted to prove **3)** whether the *OptiPrep™* method *(30, 31)* is a suitable method to obtain highly pure and RNA-containing thrombocyte isolates that are usable for qPCR studies.

Chapter 1. The *OptiPrep™* method is an appropriate method to obtain high-pure, RNA-containing thrombocyte isolates, that are suitable for gene expression studies

1.1 Introduction

Thrombocyte isolation from canine whole blood was described once before by *Trichler et al. 2013.(32)* The isolation technique used, was derived from human medicine: the *OptiPrep™* density barrier method by *J.M. Graham, 2002. (30, 31)* In this study the blood sample was centrifuged for 15 minutes and the purity was 99.47 ± 0.21 % (n= isolations?, dog number?). To decrease the leukocyte contamination they used dynabeads, but then the purity decreased to 98.84 ± 0.03 %. Indicating thrombocyte loss.

In our study we were planning to perform measurements on platelets and therefore we also used the *OptiPrep™* method. We firstly wanted to detect TF protein by use of immunocytochemistry. Secondly, by use of qPCR we would like to know, if TF protein is present, whether platelets also contain TF mRNA. In order to increase the likelihood that we measured thrombocyte associated TF mRNA and not monocyte/neutrophil associated TF mRNA, our study objective was to obtain high-pure and RNA-containing thrombocyte isolates, using the *OptiPrep™* method. We would like to create high-pure thrombocyte isolates without the use of dynabeads, in order to prevent thrombocyte loss.

1.2 Materials and methods

1.2.1 Blood collection

Blood collection was performed by venipuncture of the *Vena Jugularis*. If the dog was not co-operative or the medical situation did not allow it, the blood collection was performed by venipuncture of the *Vena Cephalica*. We needed 1 – 3 mL EDTA anticoagulated blood samples and were processed within 4 hours after blood collection. This in order to prevent RNA degradation *(33)* and excessive platelet activation *(34)*, which both could affect our measurements.

1.2.2 Patient selection

Eleven healthy dogs are compared to 24 diseased dogs. The diseased dogs were referred to the intensive care unit of Utrecht University Clinic for Companion Animals between October 2014 and April 2015. We divided the sick dog population into five groups (I – V), with 5 dogs in each group (with exception of one group that contained 3 dogs). The grouping of dogs was based on the presence of activated coagulation in these patients.

Group I dogs were diagnosed with cIIMHA and were our main interest of the current study. We compared cIIMHA dogs (n=12) with other diseased groups that surely had also activated coagulation, because at the moment cIIMHA dogs are very scarcely brought to our clinic. Additionally, we were also interested in the compare and overlap between cIIMHA dogs and the other four diseased groups. The additionally groups were: group II dogs suffering from disseminated intravascular coagulation (DIC; n=5); group III dogs have had a surgical intervention (SInt; n=3); group IV dogs were suffering from a tumour (Tum; n=5) and group V dogs were suffering from sepsis (Se; n=5). The grouping is based on earlier work of *Piek et al. 2011.(9)*

The 11 healthy control dogs comprised of 8 experimental dogs and 3 employee owned dogs. The blood collection procedure was approved according to Dutch legislation.

The criteria we used to create each of our diseased groups were as follows. Dogs with cIIMHA did not have an underlying cause of the immune mediated haemolytic anaemia (IMHA) and did have an haematocrit (Ht) of < 0.30 L/L, a positive coombs test and/or spherocytosis. Dogs with DIC were required to have ≥ 2 of the following criteria: thrombocytopenia, abnormal PT and/or APTT, hypofibrinogenemia, low plasma AT_{III} activity, increased D-dimer concentration. The patients that underwent

SInt necessitated intensive care and did not have DIC and/or Se. Those dogs suffering from Tum were necessitated of having cytological or histological evidence of neoplasia and also have evidence of DIC (≥ 2 criteria). Dogs in the Se group were diagnosed by either cyto-, histo- or microbiological confirmation of infection together with ≥ 2 of the following criteria: hypo- (< 37.8 °C) or hyperthermia (> 39.4 °C), tachycardia (> 140 BPM), tachypneu (> 20 RPM), leucopenia (< 6 x 10^9/L) or leucocytosis (> 16 x 10^9/L), > 3 % bands.

Our control group consisted of healthy dogs and before collecting the blood samples they never had been abroad before, did not receive any medication or vaccination in the last month and neither were sick at the moment or have been sick in the last 6 months.

1.2.3 OptiPrep™ method

To isolate thrombocytes from whole blood we used the *OptiPrep™ method* by *Graham. 2002*. *(31, 32)* Firstly, we prepared 100 mL diluent, which is necessary to create the appropriate density of 1.063 g/mL, by mixing it with the *OptiPrep™* stock. The diluent consisted of 0.85% NaCl, 20 mM Hepes, 1 mM NaOH (pH 7.4) and 1 mM EDTA. It was necessary to mix the *OptiPrep™* stock well, before use.

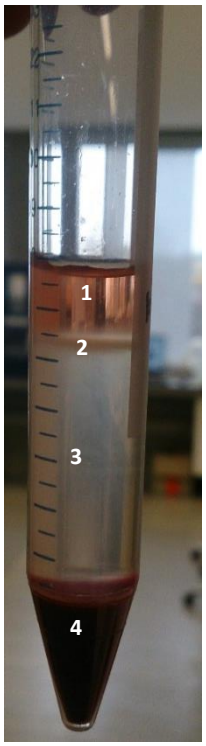

Fig. 1 Distinct layering is observed after centrifuging with OptiPrep™ solution. 1: clear layer; 2: cloudy, platelet rich, layer; 3:turbi layer; 4:pellet.

To process one EDTA anticoagulated whole blood sample, we firstly prepare the required density solution of ρ 1.063 g/mL by adding 1 mL OptiPrep™ stock with 4,4 mL OptiPrep™ diluent in a 15 mL plastic centrifuge tube and mixed it well. Thereafter, we added a minimum of 1 mL and a maximum of 3 mL whole blood sample by gently pouring it on top of the density barrier. The tube was then centrifuged at 350*g* for 30 min. at 20 °C in a swinging-bucket rotor. The centrifuge (*Hettich, Rotina 48R*) was set to decelerate without the brake to prevent disturbing the distinct cell layers.

After centrifuging, a distinct layering is observed (Fig. 1).The cloudy second layer consists of the platelets of interest and had a volume of almost 1 mL. We collected this 1 mL platelet rich layer into a 1,5 mL Eppendorf tube.

To control the purity (i.e. the % leukocyte contamination) of our platelet rich sample we used the haematology analyser (*Advia® 1120i haematology analyser, Siemens*). We performed a complete blood count analysis to check the platelet and leukocyte count. To calculate the purity of the sample we used the following formula: 1 – (leukocytes / thrombocytes) x 100 %. The unit used for the leukocytes and thrombocytes was 10^9 cells / litre blood.

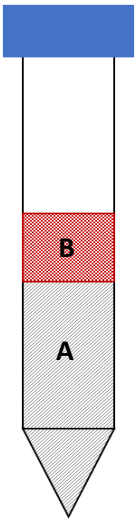

Fig. 2 A 15 mL centrifuge tube with the density barrier of 1.063 g/mL (A) and the blood sample on top (B).

1.2.4 Thrombocyte parameters

To determine whether the thrombocyte parameters indeed differed we measured those in whole blood, using the ADVIA® 2120i: mean platelet mass (MPM), the mean platelet volume (MPV) and mean platelet concentration (MPC). The platelet parameters were measured in order to observe their changes during time. Because it is known that dogs suffering of cIIMHA have an increase in mainly the MPV and the MPM. The MPC remains within the reference range, most often.

1.2.5 RNA isolation

We used the RiboPure® Blood Kit (Ambion®) and performed the RNA isolation according to the manufacturers protocol. From our total 1 mL platelet rich plasma, we picked 500 µL to perform RNA isolation. Those 500 µL platelet samples were processed with 1.0 mL RNAlater® solution, centrifuged (*Hettich, Mikro 22*) at maximum speed for 1 minute and the supernatant removed prior to storage at
-20 °C.

When continuing the RNA isolation procedure, we picked our pellet samples out the of the freezer and added 800 µl *Lysis Solution* together with 50 µl *Sodium Acetate Solution*. After this we also added 500 µl *Acid-Phenol: Chloroform*, vortexed for 30 sec. and incubated for 5 min. After incubation the tube is vortexed at maximum speed (13200 rpm) for 1 min. to separate the organic and aqueous phases. The 1 mL RNA containing aqueous phase was transferred to a new 2 mL tube and 600 µl *100% ethanol* was added. The tube was vortexed.

A new 2 mL tube with a filter cartridge was picked and the sample was loaded onto this filter, 700 µl at a time. After every loading, the tube was centrifuged at maximum speed for 30 sec. The flow-through was discarded.

The filter was washed with 700 µl *Wash Solution 1* and centrifuged at maximum speed for 30 sec. thereafter two additional washes were performed with *Wash Solution 2/3 - 100% ethanol-mixture* and each time centrifuged at maximum speed for 30 sec. The last time was centrifuged for one extra time for 1 min. to remove residual fluid from the filter. The flow-through was discarded.

The filter was transferred to a new 2 mL collection tube and 2x 10 µL *Elution Solution* (pre-heated to 75 ^{o}C) was added directly onto the membrane and incubated for 20 sec. Thereafter the tube was centrifuged at maximum speed for 1 min. The 20 µL RNA containing Elution Solution was stored on ice prior to DNAse treatment.

To the 20 µL RNA sample, 1 µL *20x DNase I Buffer* and 1 µL *DNase I Enzyme (8U/µL)* was added to remove contaminating genomic DNA and was incubated for 30 min. at 37 ^{o}C.

After incubation, 4 µL *DNase Inactivation Reagent* was added to inactivate the *DNase I Enzyme* and the tube was vortexed. After the visible separation of the aqueous and organic phase (a small pellet), the aqueous phase was transferred to a new 1,5 mL tube. The pellet was discarded. The 20 µL RNA rich sample was stored at -80 ^{o}C.

The final volume of the eluted RNA was 20 µL. The manufacturers protocol, however, uses 100 µL elution solution, but the RNA concentration of the pilot platelet samples, measured with the NANOdrop, was very low (average of 4.75 ng/µl). So, we had to concentrate our RNA yields more in order to increase the success rate of qPCR, by decreasing the volume of the *Elution Solution*.

1.2.6 cDNA synthesis

To perform cDNA synthesis we used the protocol according to the manufacturers of the *iScript cDNA Synthesis Kit* (*Biorad*®). The maximum reaction volume was 20µL and comprised of the master mix + the sample. The ‘master mix‘ consisted of 4 µL *5x iScript Reaction Buffer* and 1 µL *iScript Reverse Transcriptase*. The input of platelet RNA was set to 100ng. So, we picked the maximum allowed sample volume of 15 µL, in the case the RNA concentration of the sample was < 7 ng/µL. For the samples with ≥7 ng/µL we calculated the amount of sample we needed and supplemented with nuclease free water to end up with 15 µL. The cDNA synthesis protocol was as follows: 5 min 25 ^{o}C, 30 min 42 ^{o}C, 5 min 85 ^{o}C and hold on 4 ^{o}C. We end up with a cDNA containing volume of 20 µL. Before using cDNA for qPCR/PCR we had to dilute the sample 10x by adding 180 µL mQ. After this dilution step the cDNA samples are ready to perform qPCR/PCR.

1.2.7 qPCR reference genes expression [MyiQ™2 Two Colour Real-Time PCR Detection System]

To check whether the *OptiPrep™* method results in RNA-containing thrombocyte isolates we used qPCR to determine the expression of the reference genes GAPDH and RSP18.The presence of reference genes will mean that RNA is present and that the RNA-isolation was successful.

1.3 Results

1.3.1 *OptiPrep™* method

After centrifuging for 30 min. a distinct layering is observed (Fig. 1). Our *OptiPrep™* pilot studies revealed that the second cloudy layer, as also confirmed by others *(31, 32)*, contained statistically significant the highest platelet count. However, this layer was not the purest one. Moreover, in terms of platelet purity there was no significant difference between the three distinct layers (i.e. clear, cloudy and turbid layer) (Fig. 4). So, for making a decision to obtain the most suitable platelet sample we decided, based on the mentioned information above, not to focus on the highest purity, but on the

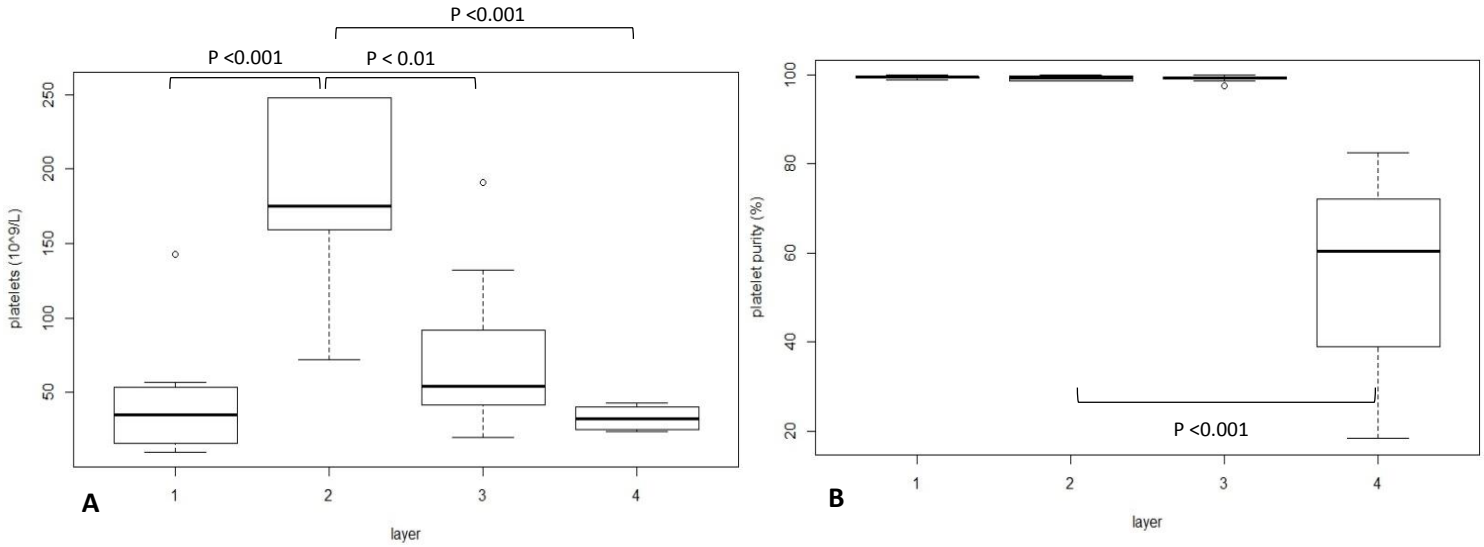

Fig. 4 Result analysis after OptiPrep™ density barrier method. ***A)*** *On the y-axis the platelet count (10^9 g/L) and on the x-axis the layer type. The second cloudy layer statistically significant contained the highest platelet count compared to the other three layers.* ***B)*** *On the y-axis the platelet purity (%) and on the x-axis the layer type. The platelet purity (%) did not statistically significant differ between the three cell layers. However, the difference between the second cloudy layer and the pellet was statistically significant ($p < 0.001$). 1: clear layer; 2: cloudy platelet rich layer; 3: turbid layer; 4: pellet. o: outliers.*

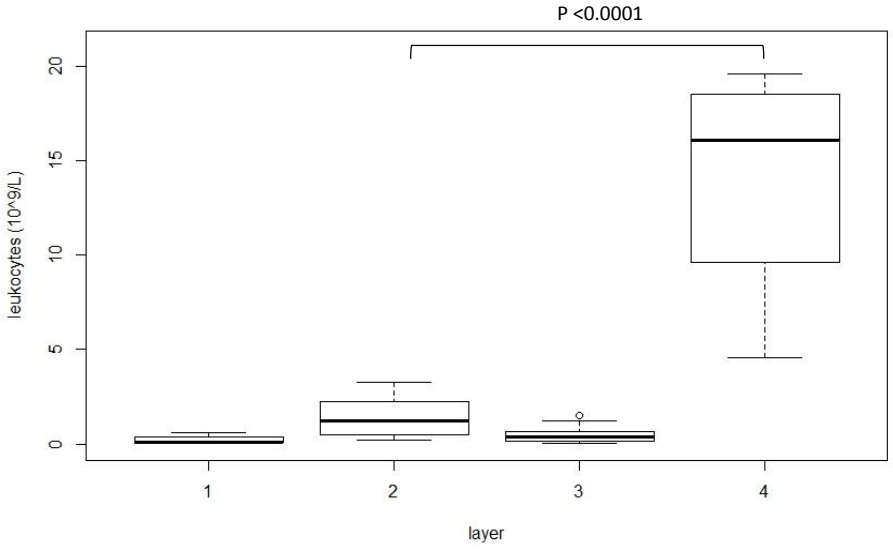

Fig. 3 . Leucocyte count within the different 4 cell layers after the OptiPrep™ method. The cloudy second layer consisted of a higher leucocyte count compared to the clear and turbid layer, but it was not significant. Significantly the most leukocytes were trapped within the pellet as was expected. 1: clear layer; 2: second cloudy platelet rich layer; 3: turbid layer; 4: pellet

highest thrombocyte count. Therefore, we could consistently collect the macroscopically visible second cloudy layer. However, as mentioned above, within the cloudy layer this highest thrombocyte count consistently paralleled with the highest, but not statistically significant, leukocyte yield (Fig. 3). Table 1 shows the different leukocyte cell types found within the second cloudy layer.

Table 1 The mean leukocyte contamination of all 66 OptiPrep™ isolations of all 5 diseased groups. Mainly lymphocytes contribute to the leukocyte contamination. Monocytes and neutrophils are both present in very low numbers (mean: <1 %).

Leucocyte type	Mean leucocytes (x10^9/L)	Percentage
Total leukocytes	11.985	100
monocytes	0.01195	0.997
lymphocytes	11.528	96.187
neutrophils	0.00812	0.678
Other leucocytes	0.02563	2.138

When starting with centrifuging for 15 minutes, which was the original centrifuge time according to the *OptiPrep™* protocol *(30, 31)*, the leukocyte contamination was even higher, compared to centrifuging for 30 min. So, to decrease the leukocyte contamination of our main thrombocyte isolates we decided to extend the centrifuge time from 15 to 30 minutes. This was successful since the leukocyte count decreased and therefore the purity increased. Our average purity of the healthy dogs (n=11) was 99.9 ± 0.06 % (n=11 isolations) and our average purity of the diseased dogs (n=30) was 98.0 ± 1.0 % (n=66 isolations). The average purity of the healthy dogs in the study of *Trichler et al. 2013*, centrifuging for 15 minutes, was 99.47 ± 0.21 % (n= isolations?, dog number?).

We performed microscopic examination of a sample smear combined with analysis by the ADVIA® 2120i haematology analyzer to reveal which type of leucocyte is actually in our target sample and whether these white blood cells are capable to interfere with our PCR/qPCR results (i.e. containing TF mRNA) or not. 2 shows the mean amount of the leukocytes (monocytes, lymphocytes and neutrophils) that are measured with the haematology analyzer. The lymphocytes were the largest and almost only leucocyte proportion in our target sample. Monocytes and neutrophils were on average < 1% present within our thrombocyte isolates.

1.3.2 Thrombocyte parameters

The ADVIA® measurements showed that especially in the beginning of the disease the MPV is above its reference range. Also the MPM is above its reference range, but compared to the MPV it relatively turns faster back to normal (Fig. 5). In contrast, the MPC did not differ from its (very broad) reference range (see the Annex for the linear graphs).

1.3.2 RNA isolation

To check the concentration of RNA (in ng/µL) within our thrombocyte samples, we used the NANOdrop. The mean concentrations of RNA in the pilot samples (n= 33) was: 4.75 ng/µL. The RNA concentration in the platelet rich samples was very low, so we decided to concentrate the RNA concentrations more in order to increase the success rate of qPCR. Therefore, the RNA of the main samples were eluted in 20 µL elution solution. The mean RNA concentration became 2.6x higher: 12.34 ng/µL (n=40), but that is still very low.

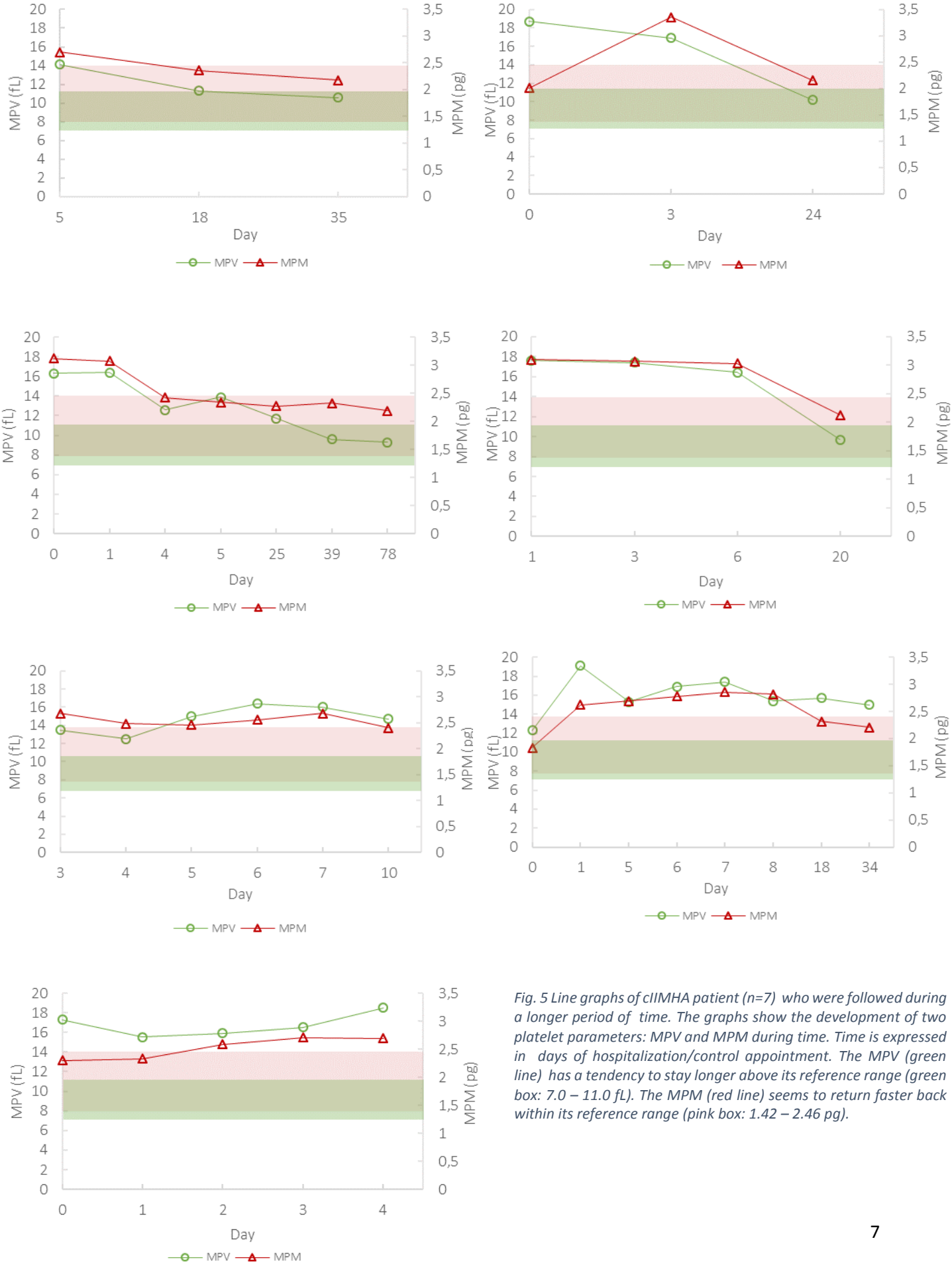

Fig. 5 Line graphs of cIIMHA patient (n=7) who were followed during a longer period of time. The graphs show the development of two platelet parameters: MPV and MPM during time. Time is expressed in days of hospitalization/control appointment. The MPV (green line) has a tendency to stay longer above its reference range (green box: 7.0 – 11.0 fL). The MPM (red line) seems to return faster back within its reference range (pink box: 1.42 – 2.46 pg).

1.3.3 qPCR reference genes expression [MyiQ™2 Two Colour Real-Time PCR Detection System]

In the platelet rich samples both reference genes GAPDH and RSP18 were expressed. So, with this information we could conclude that the samples contained RNA and that the RNA isolation was successful. Also we could say that the thrombocyte isolates, obtained by the *OptiPrep™* method, is a suitable method to obtain thrombocyte isolates that are usable for gene expression studies.

1.4 Discussion

The isolation of thrombocytes using the *OptiPrep™* method does not always result in 100% pure thrombocyte isolates. Thus, resulting in an unavoidable leukocyte contamination. The leukocyte contamination within our 66 platelet samples of all 5 disease groups are shown in 2. Mainly lymphocytes contribute to the leukocyte contamination and since lymphocytes are not known to contain TF mRNA nor express TF protein, these white blood cells will not influence our (q)PCR analysis. However, there were some samples that, besides lymphocytes, also contained a few monocytes and/or neutrophils (< 1%). These samples are obtained from dogs with (severe) leukocytosis and/or (severe) thrombocytopenia. Despite the presence of both TF producing white blood cells, we assumed that the very low numbers of both white blood cells, but mainly the monocytes, will probably not influence our gene expression study. Moreover, it also has been shown that TF measurement in monocytes is really difficult, because those cells are quickly activated when they come into contact with foreign surfaces (like blood collection tubes, needles etc.).*(31)* So, it is unclear whether monocyte contamination will influences qPCR results.

Thrombocytopenia and leukocytosis negatively influences the platelet isolation results. In our main experiment the average platelet purity of all isolations of all five groups was 98% (n=66 isolations). But after exclusion of 9 isolations with a purity of 98% or less, the average purity became 99.7%. All nine dogs had in fact (severe) thrombocytopenia (average of 77.75 x 10^9 thrombocytes/L) with or without (severe) leukocytosis (average of 65.78 x 10^9 leukocytes/L).

The use of dynabeads results in thrombocyte loss and are also not necessary to create high pure thrombocyte isolates. In our study we centrifuged for 30 minutes which resulted in an average purity of 99.9 ± 0.06 % (n=11 isolations, 11 dogs). In the study of *Trichler et al. 2013(32)* the average purity after centrifuging of 15 min. was 99.47 ± 0.21 % (n= isolations?, dog number?). They used dynabeads to remove additional leukocytes and claimed that the use of dynabeads did not result in extra thrombocyte loss. However, after using dynabeads their average purity decreased to 98.84 ± 0.03 %. Therefore we decided not to use dynabeads to decrease the leukocyte contamination, but to extent the centrifuge time from 15 to 30 min.. That was successful, since our sample purities increased and the purity of our healthy dogs was even higher compared to the purity of the healthy dogs in the study of *Trichler et al. 2013*.

Using the *RiboPure Blood Kit* to isolate RNA from thrombocyte isolates results in very low RNA yields. This could be explained by the fact that this RNA isolation kit is intended to use with whole blood samples. Whole blood samples are more diverse and cell-rich and therefore contain higher RNA yields, when compared to thrombocyte isolates. To solve the problem of our low RNA concentrations, we decided to decreased the advised volume of 100 µl *Elution Solution* to 20 µl. The RNA concentrations increased, but still they were not very high. Maybe the use of another RNA isolation kit will be more convenient, since decreasing of the volume of *Elution Solution* probably will not result in a far more increase of the RNA concentration.

Chapter 2. Tissue factor protein in thrombocytes from dogs, suffering from idiopathic immune mediated haemolytic anaemia

2.1 Introduction

Despite the scarce literature about veterinary research on canine platelet associated tissue factor (TF) production, in human studies there has been done a lot of research. Some studies demonstrate that TF protein is bound on the platelet cell membrane*(17-24)* whereas another study claims that activated platelets are also capable to release functionally active TF protein.*(19)* Moreover, not only activated platelets but also resting platelets are thought to contain TF protein (i.e. α-granules and in the open canicular system).*(21)*

The study objective was to prove whether thrombocytes of cIIMHA dogs indeed carry TF protein. To achieve this goal we used of immunocytochemistry (ICC) on cIIMHA thrombocytes.

2.2 Material and methods

2.2.1 patient selection

We compared 9 healthy dogs with 7 cIIMHA dogs. The sick dogs were brought to the intensive care unit of Utrecht University Clinic for Companion Animals between October 2014 and Mai 2015. The 6 experimental dogs and 3 employee owned dogs served as our control group.

The criteria we used to include cIIMHA dogs were as follows: no underlying cause of the cIIMHA is found, the Ht is < 0.30 L/L and the coombs test is positive and/or spherocytosis is present.

Our control group consisted of healthy dogs. Prior the blood collection, the dogs never had been abroad before, did not receive any medication or vaccination in the last month and neither were sick at the moment or have been sick in the last 6 months. The blood collection procedure was approved according to Dutch legislation. The owners signed an owner-consent form (see the Annex).

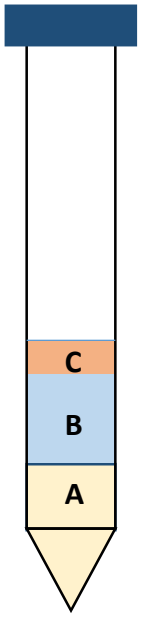

Fig. 6 A 15 mL centrifuge tube, before centrifugation, used for the isolation of monocytes and neutrophils. A: OptiPrep density layer 1.090 g/mL; B: OptiPrep density layer 1.077 g/mL; C: buffy coat. Layer A will contain the neutrophils and layer B will contain the monocytes.

2.2.2 Blood collection

(For the description see chapter 1)

2.2.3 Platelet isolation with *OptiPrep™*

(For the exact description see Chapter 1)

2.2.4 Leucocyte isolation with *OptiPrep™*

Mainly monocytes and also neutrophils are known to contain TF and are therefore a suitable positive control. So, from 9 healthy dogs (6 experimental and 3 staff owned dogs) 3 mL EDTA blood was taken and leukocyte rich samples were prepared by use of the ***OptiPrep™*** method *(35)*.

Firstly, the blood samples are centrifuged for 20 minutes at 200g at 22 °C in order to gain the buffy coat we needed. In the meantime two different OptiPrep™ density barriers were made. For the isolation of polymorphonuclear leukocytes (PMK's) we prepared a density barrier of 1.090 g/mL by adding 1 mL OptiPrep™ stock solution + 2.75 mL diluent (see 'platelet isolation' for the preparation of this diluent). To isolate the monocytes we prepared a density barrier of 1.077 g/mL by mixing 1 mL OptiPrep™ stock solution with 3.5 mL diluent.

In a 15 mL plastic centrifuge tube we firstly added the density barrier 1.077 g/mL and thereafter underlay this barrier with density barrier 1.090 g/mL. Thereafter, the buffy coat is harvested from the centrifuged blood sample and overlayered

to the density barriers (Fig. 6). The tube is then centrifuged for 25 minutes at 800*g* at 22 ^{o}C. After centrifugation two different layers will be seen. The upper layer which contains the monocytes and the lower layer which contains the PMK's. Both layers are harvested by use of a pipette and added to a 1.5 mL Eppendorf tube.

To check the method, only the first leukocyte rich sample was measured by use of the ADVIA 1210i Hematology Analyzer. The only goal of this measurement was to check whether the isolation indeed obtained leukocytes (i.e. monocytes and neutrophils), since a high sample purity was not necessary. In addition, also a sample smear was analyzed by use of the microscope to check the ADVIA measurement.

2.2.5 Erythrocyte rich sample

Since erythrocytes are not known to express and/or contain TF protein, these cells are suitable to use as a negative control. To obtain erythrocyte rich samples we used the erythrocyte pellet from the centrifuged EDTA blood sample that was used to harvest the buffy coat for leucocyte isolation. Because the pellet is a highly concentrated erythrocyte sample we diluted the erythrocytes with Phosphate Buffered Saline (PBS) in a 1:100 dilution in a final volume of 1 mL (10µL blood + 990 µL PBS) to obtain a suitable cytospin.

2.2.6 Cytospins

From each sample type (i.e. platelets, leukocytes or erythrocytes) we made a series of cytospins (*Shandon Cytospin®4; Thermo Scientific),* each consisting of four slides. Per cytospin preparation we needed 50 µL sample. One of the four slides was a May Grünwald Giemsa stain (MGG) (Tissue-Tek® DRS™2000) and used to check cell integrity, cell distribution and to differentiate the cell population. Three further slides were made for use in the ICC procedure: two slides of primary antibody (*anti-TF* or *Rabbit isotype control*) + secondary antibody. The slide with anti-TF antibody served as test. A slide with the isotype control antibody was used to control the specificity of the anti-TF antibody to tissue factor. The negative control slide was only incubated with the antibody diluent + secondary antibody and served as an internal control of the whole ICC-procedure.

2.2.7 Immunocytochemistry (ICC)

Cytospins are made around 4 days before performing the ICC procedure, were air dried and stored at room temperature (RT). Preliminary studies showed that pre-fixation with acetone damaged the cells.

When starting with the ICC procedure the slides were fixed in acetone (at room temperature) for 3 minutes. Fixation in ice cold acetone damages the blood cells, as shown in our ICC pilot studies and was therefore not suitable. The slides were air dried for about 10 minutes and thereafter washed 2x 2 minutes with PBS-Tween (100 mL *10x PBS* + 900 mL mQ + 1 mL *Tween 20* (0.1%); pH 7.4).

In order to avoid non-specific staining the endogenous peroxidase activity was blocked by adding an endogenous enzyme block (Dako Dual Endogenous Enzyme Block) and the slides were incubated for 10 minutes at RT. After this blocking step the slides were again washed with PBS-Tween for 3x 5 minutes.

To prevent high background staining the slides were incubated with normal goat serum (1:10 PBS) for 1 hour at RT. Following this step the three serial cytospin slides were differently treated. Slide one was incubated with the primary antibody (1:100 rabbit anti-human TF antibody; monoclonal IgG; ab151748 Abcam®). Slide two was incubated with the isotype antibody (1:100 anti-rabbit; monoclonal IgG; ab172730 Abcam®). The third slide was incubated with just the primary antibody diluent (Dako Antibody Diluent). All slides were thereafter incubated over night at 4 °C.

After overnight incubation the slides were rinsed in PBS-Tween for 3x 5 minutes. All slides were separately washed in order to avoid contamination of the remaining unbound antibodies. The secondary goat anti-rabbit antibody (EnVision+ System-HRP (DAB), Dako) was then added to all slides and incubated for 45 minutes at RT. Thereafter a next wash with PBS for 3x 5 minutes followed.

In order to visualize antibody binding all slides were incubated with 3,3'-Diaminobenzidine (DAB) (1 mL buffer + 1 droplet DAB) for 5 minutes. Thereafter the slides were washed again for 3x 5 minutes with mQ.

To counterstain the cells, in order to visualize the cell contours, each slide was stained for 10 seconds with haematoxylin (Haematoxylin QS-Dako) and thereafter rinsed in tap water for 10 minutes.

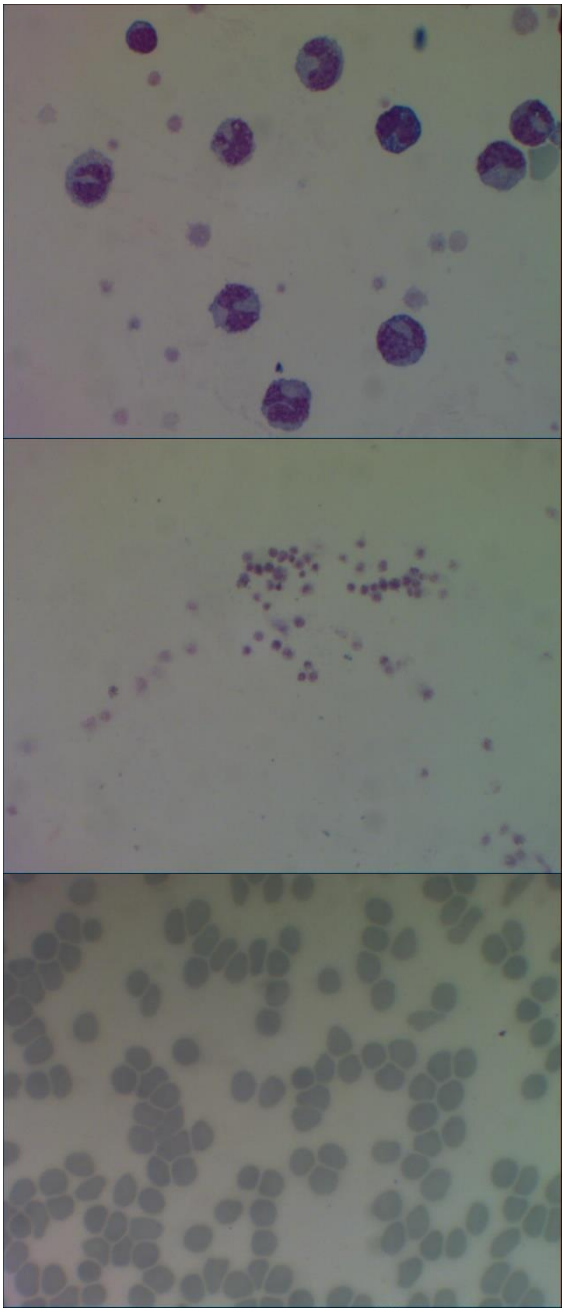

Fig. 7 The MGG staining of the cytospins showed a nice distribu-tion of intact cells. Upper photo: cytospin of leukocyte isolation; middle photo: cytospin of thrombocyte isola-tion; lower photo: erythrocytes diluted 1:100 with PBS. Microscope magnification 250x

Slides were prepared for long term storage by performing a series of dehydration by increasing the alcohol percentage from 60 % to 96 %. Each dehydration step took 5 minutes. The last two steps were treated with Xylene I and Xylene II and took 2x 3minutes. After the dehydration procedure the slides were covered with cover slips by use of vecta mount in order to store the slides for long term.

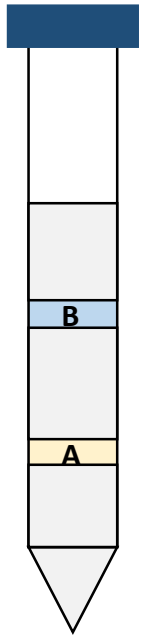

Fig. 8 OptiPrep™ leukocyte isolation. After centrifugation two distinct layers are seen. Layer A contained the neutrophils and layer B contained the monocytes

2.3 Results

2.3.1 Platelet isolation with *OptiPrep™*

(For the patient results see Chapter 1)

2.3.2 Leucocyte isolation with *OptiPrep™*

Since monocytes and neutrophils are both known to express TF protein, we isolated both cell types using OptiPrep™.*(35)*

After centrifugation distinct layering is observed. One upper and one lower layer (Fig. 8). The upper layer contained mainly monocytes and some thrombocytes and the lower layer contained neutrophils. The reason for the thrombocytes contamination within the monocytic layer is probably caused by the fact that both density barriers are close to each other (monocyte: 1.077 versus thrombocyte: 1.063 g/mL) and therefore some larger thrombocytes were trapped within the monocytic layer.

2.3.3 Erythrocyte rich sample

The MGG stained slides showed that the dilution of 1:100 was appropriate, because the erythrocytes were diffusely distributed on the slide(Fig. 7). Moreover, the correct cell type is present and the cells are intact.

2.3.4 Immunocytochemistry (ICC)

The white blood cells: mainly monocytes but also neutrophils served as a positive control since both cells are known to express TF protein. Indeed, the immunocytochemistry of these cells showed positive DAB staining (Fig. 11). As it should be, the isotype control slide and the slide without primary antibody negative control) showed no DAB staining.

The thrombocytes of cIIMHA dogs stained DAB positive (Fig. 11). So, the platelets from cIIMHA dogs contain membrane bound TF protein. Also the platelets from healthy dogs stained positive for TF protein.

To semi-quantify the thrombocyte staining and to determine whether there is a statistically significant difference between cIIMHA and healthy dogs, we used ImageJ. It was calculated what the total % cell area was that stained DAB positive, since DAB intensity cannot be quantified. That is, DAB does not follow the Beer-Lambert law which states that light absorbance is directly correlated to the concentration. So, a high DAB intensity does not automatically mean that there is also a lot TF present on the cells. The formula we used to calculate the % stained cell area: area2 / area1 x 100. 'Area2' is the DAB stained cell area and 'area1' is haematoxylin stained cell area. To determine both individual cell areas we used the ImageJ plugin 'colour deconvolution' which per photo separates DAB from hematoxylin staining. This plugin was necessary, because otherwise ImageJ was not able to distinguish between both stainings. The semi-quantification with ImageJ revealed that in case of cIIMHA dogs the % DAB positive thrombocyte cell area was statistically significant higher (79.4 %; n=7, just the samples taken at hospitalization day) compared to the % cell area of platelets from the healthy dogs (43.1 %; n=9) (Fig. 9). We further analysed, using ImageJ, whether the cIIMHA DAB staining decreases during time of hospitalization and prednisolon therapy and within what timespan. We revealed that the DAB positive cell area decreases during time and therapy, but that the progression differs individually (Fig. 10)

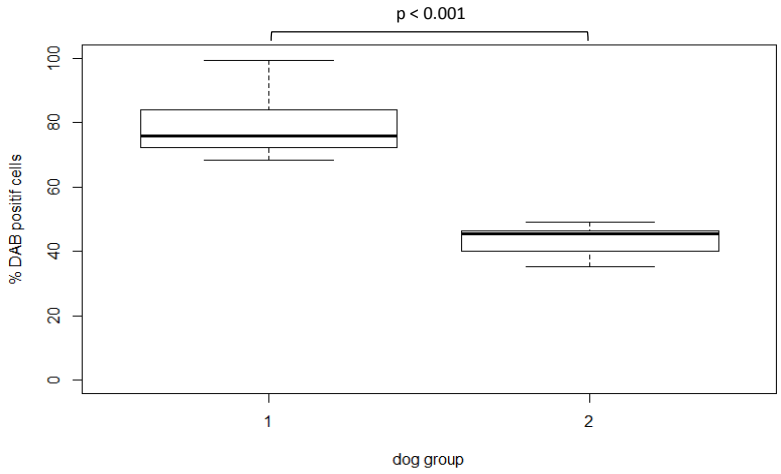

Fig. 9 Significantly higher % cell area stained TF positive in case of cIIMHA patients (group1), compared to healthy dogs (group2). cIIMHA dogs n=7; healthy dogs n=9

2.4 Discussion

It seems that thrombocytes in general carry TF, but that cIIMHA dogs have more TF-positive thrombocytes and may explain the increased risk to develop thromboembolisms. The difference between cIIMHA dogs and healthy dogs was statistically significant (Fig. 9). In human studies the presence of TF protein in thrombocytes has also been shown.*(17-24)*

It appears that the amount of TF-positive thrombocytes in cIIMHA dogs remains high during the first weeks of the disease. Also this finding may explain the increased risk of thromboembolisms

within the first two weeks of the disease. However, the exact origin of the TF protein is still unknown. There are some human studies that argue that monocytes donate TF to platelets by microvesicles.*(14-16)* Another explanation may be that the thrombocytes may have inherited TF protein from their mother cell: the megakaryocyte. Indeed, recently, a human study showed that megakaryoblasts and megakaryocytes contain TF mRNA and TF protein and that megakaryocytes donate TF protein to a subset of platelets. Even TF mRNA is donated to a subset of thrombocytes.*(25)* The same may be true in dogs and it is worth it to investigate this.

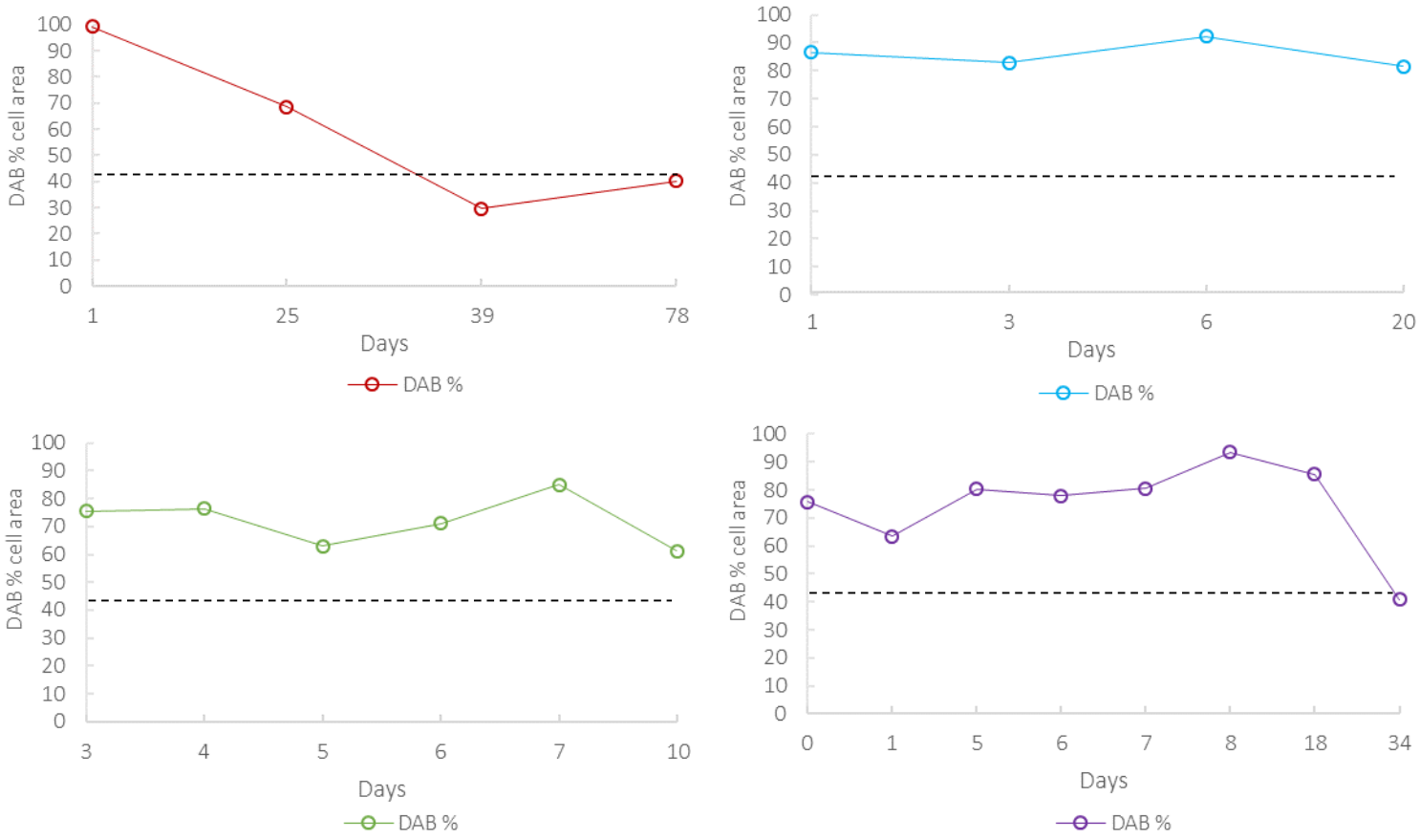

Fig. 10 Four cIIMHA patients are followed for a longer period of time. The DAB % course during hospitalization is individually shown in line graphs. It differs individually how fast the DAB staining returns to the mean DAB % of healthy dogs (black dotted line). The line graphs show that the DAB % remains high, up to 5 weeks.

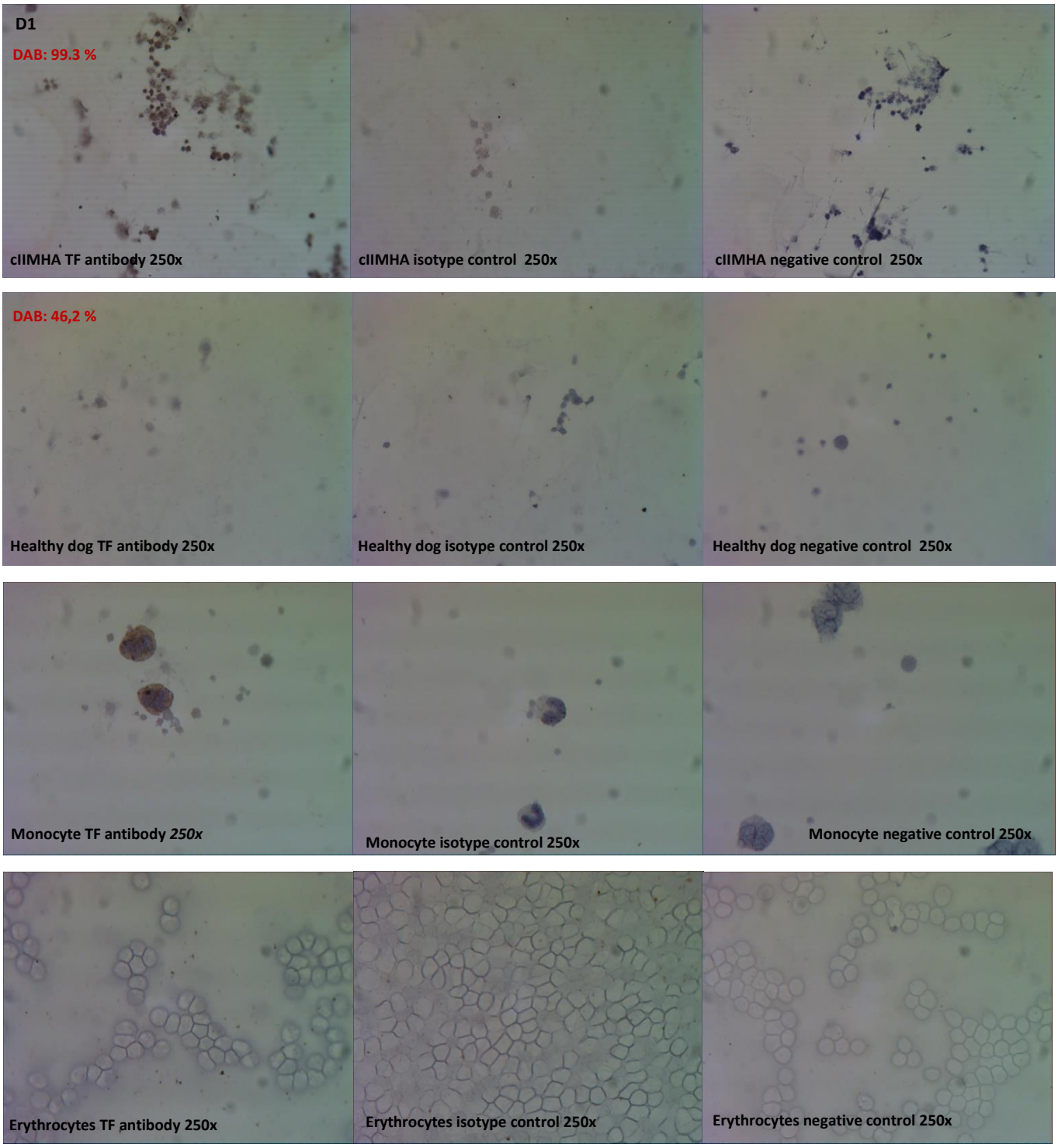

Fig. 11. ICC results of tissue factor staining using DAB. Of each row the first photo shows primary antibody against tissue factor (TF), the second photo shows primary isotype control antibody and the latter photo shows negative control (e.g. only secondary antibody). ***First three photo's****: positive DAB staining of thrombocytes form an IMHA patient at hospitalisation day 1 (D1). Percentage positive stained cell area is 99.3 %.* ***The second three photo's****: positive DAB staining thrombocytes of a healthy control dog. Percentage positive stained cell area is 46.2 %.* ***The third three photo's****: positive control cells, monocytes. As expected, the monocytes stained positive for tissue factor.* ***The last three photo's****: negative control cells, erythrocytes. As expected, the erythrocytes did not stained positive for tissue factor. Microscope magnification: 250x*

Chapter 3. The presence of tissue factor and tissue factor splice variant mRNA in thrombocytes from dogs, suffering from idiopathic immune mediated haemolytic anemia

3.1 Introduction

Dogs suffering from cIIMHA are at greater risk of dying particularly in the first two weeks after the diagnosis is made.*(1)* This high mortality risk may be associated with the development of thromboembolism (TE) *(2-6)*and/or disseminated intravascular coagulation (DIC)*(3)* and the resulting organ failure.

The exact cause of developing TE, however, is still unknown and not really investigated yet. In human studies, however, has been done a lot more research on the role of tissue factor in several prothrombotic syndromes. Most studies argue that the monocytes and neutrophils are the TF source, since those leukocytes are known to express TF. But, also some human studies has shown that thrombocytes contain TF mRNA *(20, 22, 23)* or even TF pre-mRNA.*(26-29)* However, there is still a lot of disagreement about the presence of TF mRNA in platelets which is not very surprising since platelets are anucleated cells. However, recently a human study showed the presence of TF mRNA and TF pre-mRNA in megakaryoblasts and megakaryocytes. They demonstrated that megakaryocytes donate TF mRNA, TF pre-mRNA and TF protein to a subset of platelets.*(25)* This information strengthens the ability of the presence of TF mRNA in canine thrombocytes and may play a role in the development of thromboembolisms within the first two weeks of the disease.

Besides TF, in human studies also a TF isoform is discovered. This isoform is an alternatively spliced variant of TF.*(36)* In the literature also named: alternatively spliced TF (asTF). asTF is suspected to be a pro-coagulant protein and compared to TF it is missing exon 5 and exon 6 codes for a specifically C-terminus. However, there are also human studies which argue that asTF has no pro-coagulant properties at all.*(37)* So, there is also still a lot of disagreement about the existence and function of asTF in pro-thrombotic syndromes.

TF mRNA consists of 6 exons. Exon 1 encodes the N-terminal signal sequence. The ectodomain is encoded by exon 2 – exon 5. Exon 6 encodes its transmembrane region and cytoplasmic tail. asTF mRNA, however, misses exon 5 which results in the replacement of the transmembrane and cytoplasmic part of TF (encoded by exon 6) with a unique 40 amino acid C-terminal domain which makes asTF a soluble protein.*(36)*

The exact origin of soluble asTF, however, is not known yet. Maybe platelets release asTF after being activated, because *Bogdanov et all. 2003(36)* have shown that human asTF co-localizes with platelets on thrombi and that asTF has pro-thrombotic activity when exposed to phospholipids.

Despite the scarce veterinary literature about tissue factor and its splice variant, we wanted to determine, by extrapolating information from human studies, whether canine platelets in cIIMHA do or do not contain TF mRNA and/or asTF mRNA. If so, then it may an explanation for the increased risk of developing TE in those patients.

3.2 Material and methods

3.2.1 Patient selection

Eleven healthy dogs are compared to 24 diseased dogs. The diseased dogs were referred to the intensive care unit of Utrecht University Clinic for Companion Animals between October 2014 and April 2015. We divided the diseased dog population into five groups (I – V), with 5 dogs in each group (with exception of one group that contained 3 dogs). The grouping of dogs was based on the presence of activated coagulation in these patients.

Group I dogs were diagnosed with cIIMHA and were our main interest of the current study. We compared cIIMHA dogs (n=12) with other diseased groups (n=18) that have evidence of activated coagulation. Additionally, we were also interested in the compare and overlap between cIIMHA dogs

and the other four diseased groups. The additionally groups were: group II dogs suffering from disseminated intravascular coagulation (DIC; n=5); group III dogs have had a surgical intervention (SInt; n=3); group IV dogs were suffering from a tumour (Tum; n=5) and group V dogs were suffering from sepsis (Se; n=5). The grouping is based on earlier work of *Piek et al. 2011*.*(9)*

The 11 healthy control dogs comprised of 8 experimental dogs and 3 employee owned dogs. The blood collection procedure was approved according to Dutch legislation.

The criteria we used to create each of our diseased groups were as follows. Dogs with cIIMHA did not have an underlying cause of the immune mediated haemolytic anaemia (IMHA) and did have an haematocrit (Ht) of < 0.30 L/L, a positive coombs test and/or spherocytosis. Dogs with DIC were required to have ≥ 2 of the following criteria: thrombocytopenia, abnormal PT and/or APTT, hypofibrinogenemia, low plasma AT_{III} activity, increased D-dimer concentration. The patients that underwent SInt necessitated intensive care and were not allowed to have DIC and/or Se. Those dogs suffering from Tum were necessitated of having cytological or histological evidence of neoplasia and also have evidence of DIC (≥ 2 criteria). Dogs in the Se group were diagnosed by either cyto-, histo- or microbiological confirmation of infection together with ≥ 2 of the following criteria: hypo- (< 37.8 ^{o}C) or hyperthermia (> 39.4 ^{o}C), tachycardia (> 140 BPM), tachypneu (> 20 RPM), leucopenia (< 6 x 10^9/L) or leucocytosis (> 16 x 10^9/L), > 3 % bands.

Our control group consisted of healthy dogs and before collecting the blood samples they never had been abroad before, did not receive any medication or vaccination in the last month and neither were sick at the moment or have been sick in the last 6 months.

3.2.2 Blood collection

Blood was obtained by jugular or cephalic venipuncture and directly collected into an EDTA anticoagulated blood tube. We chose EDTA because both sodium citrate and heparin anticoagulated blood were not suitable to use for our study. Compared to EDTA, sodium citrate results in a higher amount of activated platelets (i.e. p-selectin expression) without activation by a platelet agonist (PMA) and a higher amount of platelet aggregates upon stimulation with platelet agonists (PMA).*(34)* So, with sodium citrate anticoagulated blood we were not able to obtain reliable platelet parameters. In the case of heparin anticoagulated blood it is generally known that heparin up regulates AT_{III} and is therefore not reliable to in-/exclude our DIC patients in our diseased dog population, since low AT_{III} is one of the inclusion criteria to diagnose DIC.

Blood samples (1 – 3 mL EDTA) were processed by the *OptiPrep™* method and *RNAlater®* treatment within 4 hours after blood collection and without storage at 4 °C. We chose this temperature and time frame, because in veterinary literature there is a study which has shown that after four hours storage (at 4 °C) of EDTA anticoagulated canine whole blood results in a significantly increase of thrombocyte produced micro particles.*(38)* These micro particles are thought to be produced by activated thrombocytes and therefore may function as a platelet activation marker and simultaneously may also be a predictor of increased TF expression. Moreover, in human medicine it has been shown that platelet concentrates prepared from one day old whole blood significantly contain increased TF expression when compared to fresh whole blood samples. These platelet concentrates showed a further increase of TF expression after one day of storage at 4 °C, which became significant at 5 days of storage.*(17)*

After taken these two studies together it appears that handling and storage of whole blood samples at 4 ^{o}C triggers platelets to express TF and that TF expression further increases during storage of platelet concentrates.*(17)* Therefore platelets in 4 ^{o}C stored blood may have already been starting expressing TF and do not represent the most approachable *in vivo* situation of platelet TF expression.*(38)* So, we chose the safest way by processing our freshly obtained whole blood samples within 4 hours after blood collection to minimize the risk of platelet activation and the possibility of excessive TF expression.

Table 2 Both primer pairs used to detect tissue factor and alternative spliced tissue factor (asTF).

Primer pair	Sequence (5'>3')	Product size (bp)	T_A (°C)
TF		228	61.4
Forward primer	AAGGTGAACGGCACATTTCT		
Reverse primer	GGTGCACTCAATGGGACTTT		
asTF		152	60.0
Forward primer	GAAATGTTCCTCGTCATTGGA		
Reverse primer	CAAGGGCACCTTCTTTATGC		

3.2.3 Primer design

The primers were designed using the software *UGene®*. To detect TF and asTF, we designed two different primer pairs. One primer specifically for tissue factor and the second one for asTF. In Table 2 are both primers presented and in Fig. 12 is the localisation of the forward and reverse primers illustrated.

3.2.4 Thrombocyte isolation with *OptiPrep™*

(See chapter 1 for details)

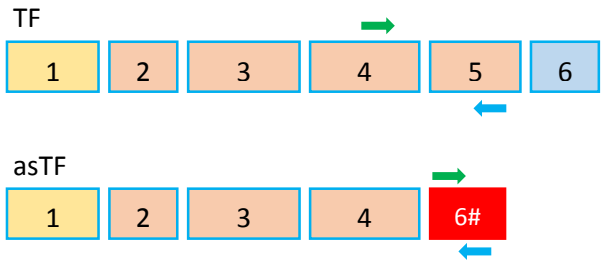

Fig. 12 Schematic illustration of the exons of TF and asTF and the attachment of the designed primers. TF comprises of 6 exons and asTF comprises 5 exons, where exon 5 misses. For TF: exon 1 (yellow) encodes the N-terminal signal sequence; exons 2 – 5 (orange) encodes the ectodomain; exon 6 (blue) encodes the transmembrane region and cytoplasmic tail. For asTF: exon 1 (yellow) encodes the N-terminal signal sequence; exon 2 – 4 (orange) encodes the ectodomain; exon 6# (red) is a unique C-terminus. Forward primer: green arrows; reverse primer: blue arrows. TF primer anneals on exon 4 and exon 5 and makes this primer pair specific for TF, since asTF lacks exon 5. asTF primer anneals within its unique C-terminal domain, encoded by asTF exon 6. That makes this primer pair specific for asTF.

3.2.5 RNA isolation

3.2.5.1 Platelets

(see chapter 1 for details)

3.2.5.2 Mammary tumour tissue

Before starting the isolation procedure we had to crush the tissue which was frozen in advance in liquid nitrogen. We used a pellet pestle to crush the cells. Thereafter we added 350 µl of the buffer RLT - ß-mercaptoethanol mixture (10 µL ß-mercaptoethanol per 1 mL buffer RLT) to the 2 mL tube which contained the crushed cells and stored it on ice. Buffer RLT contains guanidine isothiocyanate.

To isolate RNA from the crushed canine mammary tumour cells we used the *RNeasy® Mini Kit* from QIAGEN, according to the manufacturer's protocol. We added 350 µL 70 % ethanol to the crushed cells, mixed gently and centrifuged for 30 sec. at maximum speed (13200 rpm). The flow through discharged. Thereafter we added 350 µL buffer RW1, centrifuged for 30 sec. at max. speed and discharged the flow through. Buffer RW1 contains ethanol and a guanidine salt and is used as a washing buffer. It

efficiently removes biomolecules (i.e. proteins, carbohydrates and fatty acids) that are non-specifically bound to the membrane. But at the other hand it enables RNA molecules, larger than 200 bases, to remain bound to the membrane.

To remove contaminating genomic DNA, we added 80 µL DNase directly on the membrane and incubated for 15 minutes. Then another wash with 350 µL buffer RW1, centrifuging at max. speed for 30 sec. and discharging the flow through.

In order to work as clean as possible we picked a new 2 mL collecting tube and transferred the membrane to it. Then we continued the RNA isolation process with adding 500 µL buffer RPE (4 vol. 96 % ethanol : 1 vol. RPE stock solution), centrifuging at max. speed for 30 minutes and discharged the flow through. Buffer RPE is a mild washing buffer and its main function is removing salts which are still on the membrane, due to buffers used earlier in the protocol. A second time we added 500 µL buffer RPE to wash the membrane. Centrifuged for 2
min. at maximum speed and discharged the flow through.

For the second time a new 2 mL collection tube is used and the membrane is transferred to it. Then we centrifuged for 1 min. at max. speed with the cover lid open. To elute the RNA we discharged this 2 mL collection tube and picked a new 1.5 mL Eppendorf tube to which the membrane is transferred. Then we added 32 µl RNase Free Water (pure quality-tested water) directly onto the membrane, incubate for 1 minute and centrifuged for 1 minute at maximum speed. The membrane is discharged and the RNA is eluted in 32 µl RNase Free Water.

3.2.6 cDNA synthesis

(For details see chapter 1)

3.2.7 Primer optimization

Both primer pairs are tested and optimized using canine mammae tumour samples, since mammary tumours are known to express both TF *(39, 40)* and asTF. *(39)* We also used pooled canine whole blood samples from our pilot patients with activated coagulation to compare the gel electrophoresis results between both.

3.2.8 Sequencing

After optimization of the two primer pairs we wanted to know whether the PCR product indeed is TF/asTF. Both electrophoresis and sanger sequence were done to identify the TF and asTF amplicons.

3.2.9 qPCR

With use of the MyiQ™2 Two Colour Real-Time PCR Detection System we determined the presence of TF and asTF. One 96 wells qPCR plate comprised of a duplo standard line and platelet rich samples from all cIIMHA patients at the first day of blood sampling + all other coagulation positive patient groups. Another 96 wells qPCR plate was filled with a duplo standard line and the platelet rich samples from all cIIMHA dogs during time of hospitalisation.

All two plates were run in duplo, because there was not enough space for a duplo within the 96 wells plate. So, per primer pair there were 4 plates (2 x 2 duplo's). The duplo’s were also mirrored. The benefit of running a duplo in two separate 96 wells plates is that you are able to rule out technical interference of the qPCR results.

3.3 Results

3.3.1 Primer optimization

Many PCR protocols were tested. The choice was between *PlatinumTaq* or *Q5® Hot Start High-Fidelity DNA Polymerase* The optimal PCR annealing temperature for the TF primer is 55 °C (*PlatinumTaq* + 1 M Betaine, 40 cycles) and for the asTF primer 60 °C (*Q5® Hot Start High-Fidelity DNA Polymerase*, 40 cycles).

With those protocols canine mammae tumour showed PCR products for both primers. Primer pair 1 detects TF and makes a product of 228bp. Primer pair 2 detects asTF and makes a product of 152bp (Fig. 13). We also tested pooled whole blood for the presence of TF and asTF and both products showed up, but asTF is very weak and therefore hard to see on the picture. It is obvious that in mammary tumour more TF is present compared to whole blood.

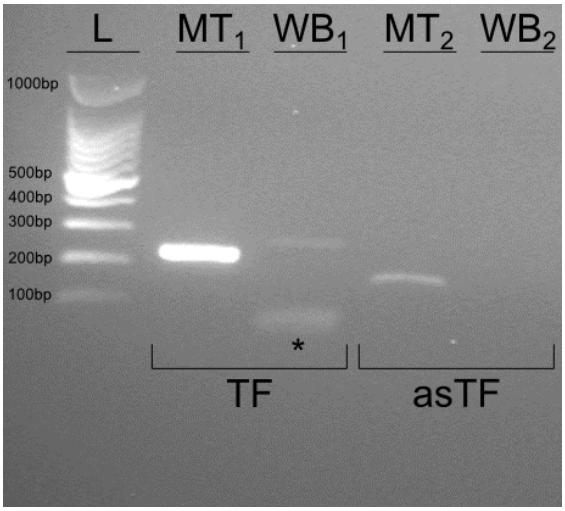

*Fig. 13 Gel electrophoresis results. L: 100bp ladder; MT_1: canine mammary tumour, TF; WB_1: whole blood, TF; MT_2: canine mammary tumour, asTF; WB_2: whole blood, asTF. *: primer dimer; $_1$: TF primer (amplicon 228bp); $_2$: asTF primer (amplicon 152bp).*

3.3.2 Sequencing

The PCR products formed in mammae carcinoma samples were sequenced. The sequencing results showed that both TF and asTF are produced. That makes them specific for the detection of tissue factor mRNA and are therefore suitable to use in our platelet samples. (See the Annex for the sequence data)

3.3.3 qPCR

Tissue factor was detectable in some cIIMHA platelet samples (n=5) and in one of the tumour patients (n=1), diagnosed with malignant histiocytosis in liver and spleen (see the Annex for the melt curves). In all these patients the normalized Cq-values were high. That means that the TF gene expression is low. The healthy control dogs all were negative to TF and asTF expression. asTF was not detectable in our platelet samples at all. However, the internal positive control, canine mammary tumour, was positive for asTF and TF as well. The Cq-value for TF expression in mammary tumour was lower compared to the cIIMHA and tumour patient. That means that the TF expression in mammary tumour is relatively higher. The Cq-value of asTF expression in mammary tumour was higher than the Cq value of the TF expression. So, in mammary tumours the expression of asTF is lower than TF (Table 3).

The Cq-values were normalized to input cDNA*(41)*, because no reference genes were used. However, this method is not the most reliable one, because you do not correct for the type of RNA.

3.4 Discussion

As the results show, there is some evidence for the presence of TF in platelets of dogs that suffer from cIIMHA (n=5) and cancer (n=1). Although, the TF concentration is very low in platelets of those patients when compared to canine mammary tumour (Table 3). In 5 out 6 of the TF positive platelet samples there was some contamination with monocytes. These cells are known to express TF and maybe also asTF. So, it may be that the TF expression measured by qPCR is originated from the monocytes. However, that does not explain why the monocyte negative sample was also positive to TF. Moreover, if monocytes were indeed the only TF source than you may expect that all other samples which were far more contaminated with monocytes were positive to TF and/or asTF, but that was certainly not the case. So, with that information it may argued that monocytes did not influence our qPCR results and that TF mRNA originates from thrombocytes.

The reason that, as we did not fully expect, only some and not all cIIMHA patients were positive to TF may be explained by the very low RNA concentrations within our sample. Trying to use another RNA isolation kit, unlike the currently used *RiboPure® Blood Kit*, might be useful, in order to obtain higher RNA concentrations and increasing the successrate of qPCR. Another explanation may be that recently, in human research, it has been shown that only a subset of thrombocytes receives TF mRNA from the megakaryocyte. So, due to our small patient size it is possible that we missed those patients that have had high amounts of TF mRNA positive platelets. Therefore resulted in hardly detectable TF mRNA concentrations by qPCR. Moreover, that study also revealed that platelets mainly received TF pre-mRNA, rather than TF mRNA, and that thrombocytes that received TF protein contained less TF mRNA. So, this could explain the results of high % DAB-positive thrombocytes, but low/negative results with qPCR.

The reason why we were not able to measure asTF mRNA at all, is probably due to the fact that this soluble TF variant is not produced by platelets. Or it is produced by platelets, but with platelet activation, this asTF mRNA is shed within platelet derived micro-particles. These micro-particles may get lost during the several dilution and washing steps and asTF is therefore not detectable anymore with qPCR. Indeed, recently a veterinary study showed that dogs with IMHA contain TF positive micro-particles *(42)*. But that study did not reveal whether it was TF or TF splice variant. They also tested dogs with primary and secondary IMHA and it were mainly the dogs with secondary IMHA that did have those TF positive micro-particles.

Table 3. This table shows the Cq-values of the positive qPCR samples. #: the Cq-values are normalized to input cDNA since no reference genes are used.(41) The cDNA input (in ng) as starting quantity for qPCR are also shown.

Gene	Patient	Cq-value	Normalized Cq-value[#]	cDNA input qPCR (ng)
TF	IMHA1	30.16	34.48	2
	IMHA2	30.87	35.19	2
	IMHA3	30.84	35.16	2
	IMHA4	31.98	35.08	0.86
	IMHA5	29.71	34.03	2
	tumour	29.73	33.51	1.38
	mammary tumour	21.43	22.43	2
asTF	mammary tumour	28.01	28.9	2

Chapter 4. General discussion and conclusion

The *OptiPrep™* isolations in our healthy control group were far more pure, compared to the study of *Trichler et al. 2013.(32)* In our study we tried to decrease the leukocyte contamination without the use of dynabeads, since the purity in the study of *Trichler et al. 2013* decreased, compared to just centrifuging for 15 min.. Therefore, we decided to extent the centrifuge time from 15 min. to 30 min. and this was really successful, since our purity was higher, compared to the study of *Trichler et al. 2013*. In our diseased group we revealed that leucocytosis and/or thrombocytopenia negatively influences platelet sample purity. So, in those patients it might be useful trying to increase the purity by centrifuging > 30 min, but with prevention of excessive thrombocyte activation, since this may influence gene expression results. Indeed, the *OptiPrep™* method results in RNA-containing thrombocyte isolates, because the reference genes are expressed. In conclusion, *OptiPrep™* is a suitable method to obtain highly-pure and RNA-containing thrombocyte isolates that are usable in gene expression studies.

The isolation of thrombocytes, using the *OptiPrep™* density barrier method, does not always result in 100% pure platelet rich samples. Thus, resulting in an unavoidable leukocyte contamination and therefore a risk of 'false' TF expression measurement by qPCR (i.e. measurement of monocyte dependent TF expression). However, our qPCR results showed that there is some evidence for the presence of TF mRNA in cIIMHA patients and in tumour patients as well. Although not all TF mRNA positive samples were 100 % pure, also the samples with no monocyte contamination are positive to TF mRNA. So, we may assume that in our study the average monocyte contamination of <1% did not influence the qPCR results and that TF mRNA originates from thrombocytes. Additionally, recently a human study *(25)* demonstrated the donation of TF mRNA to thrombocytes by the megakaryocytes. This strengthens our qPCR results, because the same might be true in dogs since dogs and man do not differ very much from one another. The reason for the very low numbers of TF mRNA positive thrombocyte isolates may be caused by the very low RNA concentrations within our thrombocyte samples. Therefore the qPCR machine was not able to detect enough TF mRNA to generate a strong signal, which resulted in very high numbers of cycles. Another RNA isolation kit, unlike the used *RiboPure® blood kit* in our study, may be useful to try. Because this RNA isolation kit is intended to use with whole blood samples and not with only thrombocyte isolates, which are just a little part of all the different cells within blood. Therefore, it probably results in very low RNA concentrations.

The % DAB-positive cell area of thrombocytes from dogs suffering from cIIMHA, stained statistically significant more DAB positive compared to the healthy control dogs. So, it may argued that dogs that suffer from idiopathic immune mediated haemolytic anemia, have more thrombocytes that carry TF protein. However, the exact origin of thrombocyte associated TF is still debatable. They may have received TF protein by microvesicles originating from activated monocytes, or from the megakaryocytes. Indeed, recently a human study showed that a subset of thrombocytes receives functionally active TF-protein from their mother cell: the megakaryocyte.*(25)* Since this human study also demonstrated the donation of TF mRNA to thrombocytes, in humans it already has been demonstrated that thrombocytes are able to produce their own functionally active TF protein from TF mRNA. Further research is warranted to demonstrate if the same is true in dogs.

Chapter 5. Further research suggestions

Since recently a human study *(25)* demonstrated the donation of TF protein and TF mRNA by megakaryocytes to thrombocytes, it may be very interesting to investigate this in dogs too. It will also strengthen the presumption of the presence of thrombocyte-associated, other than monocyte donated, TF protein in canine thrombocytes. However, since megakaryocytes are located in the bone marrow, it would be too invasive and ethically irresponsible to puncture the bone marrow for harvesting the megakaryocytes. The easiest way would be using a canine-megakaryoblastic cell line that is able to differentiate into megakaryocytes.

In our study there has been evidence for the presence of TF mRNA in cIIMHA thrombocytes. But in order to obtain more reliable data, a repeated qPCR experiment with use of reference genes is warranted.

To increase the sample purity, in cases of blood samples with severe thrombocytopenia and/or leucocytosis, it may be useful to centrifuge more than 30 minutes, but preventing excessive thrombocyte activation.

Because we had a small sample size in immunocytochemistry, it may be worth it to repeat this experiment with a larger sample size, in order to get more data.

To increase the RNA concentration within the thrombocyte isolates and therefore increasing the successrate of qPCR, it may be necessary to use another RNA-isolation kit.

Chapter 6. Acknowledgements

I would like to thank my both supervisors Christine Piek and Louis Penning for the opportunity to be a part of their research subject and their support during my minor research project. Also would I like to thank the analysts of the UVDL haematology laboratory and especially Martin van Leeuwen for sharing all his knowledge and willingness to help me with my project. The analysts of the JDV laboratory I also want to thank for their knowledge and help and especially Manon Vos-Loohuis for her support and knowledge on the part of genetics.

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Literature

1. C. J. Piek *et al.*, Idiopathic immune-mediated hemolytic anemia: treatment outcome and prognostic factors in 149 dogs. *Journal of Veterinary Internal Medicine.* **22**, 366-73 (2008).

2. R. K. Fenty, A. M. Delaforcade, S. P. Shaw, T. E. O. Toole, Identification of hypercoagulability in dogs with primary immune-mediated hemolytic anemia by means of thromboelastography. *J. Am. Vet. Med. Assoc.* **238**, 463-467 (2011).

3. A. Carr, D. Panciera, L. Kidd, Prognostic factors for mortality and thromboembolism in canine immune-mediated hemolytic anemia: a retrospective study of 72 dogs. *Journal of Veterinary Internal Medicine.* **16**, 504-9 (2002).

4. J. C. Scott Moncrieff, N. G. Treadwell, S. M. McCullough, M. B. Brooks, Hemostatic abnormalities in dogs with primary immune-mediated hemolytic anemia. *J. Am. Anim. Hosp. Assoc.* **37**, 220-7 (2001).

5. M. K. Klein, S. W. Dow, R. A. Rosychuk, Pulmonary thromboembolism associated with immune-mediated hemolytic anemia in dogs: ten cases (1982-1987). *J. Am. Vet. Med. Assoc.* **195**, 246-50 (1989).

6. P. M. McManus, L. E. Craig, Correlation between leukocytosis and necropsy findings in dogs with immune-mediated hemolytic anemia: 34 cases (1994-1999). *J. Am. Vet. Med. Assoc.* **218**, 1308-13 (2001).

7. D. J. Weiss, J. L. Brazzell, Detection of activated platelets in dogs with primary immune-mediated hemolytic anemia. *Journal of Veterinary Internal Medicine.* **20**, 682-686. 27 ref (2006).

8. S. Falati *et al.*, Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. *J. Exp. Med.* **197**, 1585-1598 (2003).

9. C. J. Piek *et al.*, High intravascular tissue factor expression in dogs with idiopathic immune-mediated haemolytic anaemia. *Vet. Immunol. Immunopathol.* **144**, 346-54 (2011).

10. A. Mälarstig, A. Siegbahn, The intersubject variability of tissue factor mRNA production in human monocytes-relation with the toll-like receptor 4. *Thromb. Res.* **120**, 407-413 (2007).

11. R. P. Rivers, W. E. Hathaway, W. L. Weston, The endotoxin-induced coagulant activity of human monocytes. *Br. J. Haematol.* **30**, 311-316 (1975).

12. N. Maugeri *et al.*, Human polymorphonuclear leukocytes produce and express functional tissue factor upon stimulation. *Journal of Thrombosis and Haemostasis.* **4**, 1323-1330 (2006).

13. B. N. Y. Setty *et al.*, Tissue factor-positive monocytes in children with sickle cell disease: Correlation with biomarkers of haemolysis. *Br. J. Haematol.* **157**, 370-380 (2012).

14. U. Rauch *et al.*, Transfer of tissue factor from leukocytes to platelets is mediated by CD15 and tissue factor. *Blood.* **96**, 170-175 (2000).

15. P. L. A. Giesen *et al.*, Blood-borne tissue factor: Another view of thrombosis. *Proceedings of the National Academy of Sciences of the United States of America.* **96**, 2311-2315 (1999).

16. M. A. Sovershaev, E. M. Egorina, B. Østerud, J. -. Hansen, Evidence for direct transfer of tissue factor from monocytes to platelets in whole blood. *Blood Coagulation and Fibrinolysis.* **23**, 345-350 (2012).

17. A. Vignoli *et al.*, Tissue factor expression on platelet surface during preparation and storage of platelet concentrates. *Transfusion Medicine and Hemotherapy.* **40**, 126-132 (2013).

18. A. Zillmann *et al.*, Platelet-associated tissue factor contributes to the collagen-triggered activation of blood coagulation. *Biochemical and Biophysical Research Communications.* **281**, 603-609 (2001).

19. F. A. Siddiqui, H. Desai, A. Amirkhosravi, M. Amaya, J. L. Francis, The presence and release of tissue factor from human platelets. *Platelets.* **13**, 247-253 (2002).

20. M. Camera *et al.*, Platelet activation induces cell-surface immunoreactive tissue factor expression, which is modulated differently by antiplatelet drugs. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **23**, 1690-1696 (2003).

21. I. Müller *et al.*, Intravascular tissue factor initiates coagulation via circulating microvesicles and platelets. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology.* **17**, 476-478 (2003).

22. O. Panes *et al.*, Human platelets synthesize and express functional tissue factor. *Blood.* **109**, 5242-5250 (2007).

23. M. Brambilla *et al.*, Tissue factor in patients with acute coronary syndromes: Expression in platelets, leukocytes, and platelet-leukocyte aggregates. *Arterioscler. Thromb. Vasc. Biol.* **28**, 947-953 (2008).

24. M. Camera, M. Brambilla, V. Toschi, E. Tremoli, Tissue factor expression on platelets is a dynamic event. *Blood.* **116**, 5076-5077 (2010).

25. M. Brambilla *et al.*, Human megakaryocytes confer tissue factor to a subset of shed platelets to stimulate thrombin generation. *Thromb. Haemost.* **114**(2015).

26. M. M. Denis *et al.*, Escaping the nuclear confines: Signal-dependent pre-mRNA splicing in anucleate platelets. *Cell.* **122**, 379-391 (2005).

27. H. Schwertz *et al.*, Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenecity of human platelets. *J. Exp. Med.* **203**, 2433-2440 (2006).

28. M. T. Rondina *et al.*, The septic milieu triggers expression of spliced tissue factor mRNA in human platelets. *Journal of Thrombosis and Haemostasis.* **9**, 748-758 (2011).

29. A. Gerrits, C. Koekman, T. van Haeften, J. W. N. Akkerman, Platelet tissue factor synthesis in type 2 diabetic patients is resistant to inhibition by insulin. *Diabetes.* **59**, 1487-95 (2010).

30. J. Graham, OptiPrep density gradient solutions for mammalian organelles. *TheScientificWorldjournal.* **2**, 1440-1443 (2002).

31. J. M. Graham, Isolation of human platelets (thrombocytes). *TheScientificWorldjournal.* **2**, 1607-1609 (2002).

32. S. A. Trichler, S. C. Bulla, J. Thomason, K. V. Lunsford, C. Bulla, Ultra-pure platelet isolation from canine whole blood. *BMC Veterinary Research; 2013.* **9**, (17;). 13 ref (2013).

33. J. Mueller, J. M. Rox, K. Madlener, B. Poetzsch, Quantitative Tissue Factor Gene Expression Analysis in Whole Blood: Development and Evaluation of a Real-Time PCR Platform. *Clin. Chem.* **50**, 245-247 (2004).

34. A. Moritz, B. K. Walcheck, D. J. Weiss, Flow cytometric detection of activated platelets in the dog. *Veterinary Clinical Pathology.* **32**, 6-12 (2003).

35. J. M. Graham, Isolation of human polymorphonuclear leukocytes (granulocytes) from a leukocyte-rich fraction. *TheScientificWorldjournal.* **2**, 1393-1396 (2002).

36. V. Y. Bogdanov *et al.*, Alternatively spliced human tissue factor: A circulating, soluble, thrombogenic protein. *Nat. Med.* **9**, 458-462 (2003).

37. A. N. Böing, C. M. Hau, A. Sturk, R. Nieuwland, Human alternatively spliced tissue factor is not secreted and does not trigger coagulation. *Journal of Thrombosis and Haemostasis.* **7**, 1423-6 (2009).

38. M. J. Wilkerson, W. Shuman, Alterations in normal canine platelets during storage in EDTA anticoagulated blood. *Veterinary Clinical Pathology.* **30**, 107-113. 29 ref (2001).

39. B. Kocatürk *et al.*, Alternatively spliced tissue factor promotes breast cancer growth in a ß1 integrin-dependent manner. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 11517-22 (2013).

40. T. Stokol, J. L. Daddona, L. S. Mubayed, J. Trimpert, S. K. Kang, Evaluation of tissue factor expression in canine tumor cells. *Am. J. Vet. Res.* **72**, 1097-1106. 50 ref (2011).

41. I. Rieu, S. J. Powers, Real-time quantitative RT-PCR: design, calculations, and statistics. *Plant Cell.* **21**, 1031-3 (2009).

42. L. Kidd, Procoagulant Microparticles in Dogs with Immune-Mediated Hemolytic Anemia. *Journal of Veterinary Internal Medicine.* **29**, 908-916 (2015).

Annex

A: Owner consent form

Akkoordverklaring eigenaar

Betreft: onderzoek van Imke Hennink en Christine Piek

Ten behoeve van ons onderzoek omtrent de stollingsproblematiek bij honden met idiopathische immuungemedieerde hemolytische anemie (IMHA) zijn wij op zoek naar 20 gezonde honden waarbij er minimaal 1 en maximaal 3 mL EDTA-bloed mag worden afgenomen. Uit dit volbloed worden de bloedplaatjes (trombocyten) geïsoleerd en getest op de aanwezigheid van eiwit en/of mRNA van het stollingseiwit tissue factor. We vermoeden namelijk dat de bloedplaatjes een belangrijke rol spelen in deze stollingsproblematiek. Dankzij uw medewerking kunnen de resultaten van de gezonde hondenpopulatie vervolgens worden vergeleken met de resultaten van de zieke honden.

Deze akkoordverklaring omvat de volgende punten:

De eigenaar…
- …gaat akkoord met een eenmalige bloedafname bij zijn/haar hond van min. 1 en max. 3 mL ten behoeve van het wetenschappelijk onderzoek.
- …gaat ermee akkoord dat de bloedafname in eerste instantie in de hals (*v. Jugularis*) zal plaatsvinden. Indien dit door oncoöperatief gedrag of anderszins onmogelijk blijkt, zal er uit de voorpoot (*v. Cephalica*) bloed worden afgenomen.
- … heeft toestemming gegeven om de informatie omtrent de ziektegeschiedenis van hond vrij te geven ten behoeve van het wetenschappelijk onderzoek.
- …heeft naar waarheid alle vragen omtrent de gezondheidsstatus en de ziektegeschiedenis van de hond beantwoord en de hond voldoet hiermee aan de criteria die gevraagd zijn om als gezonde hond deel te mogen nemen aan dit onderzoek.
- …is zich ervan bewust dat deelname berust op vrijwillig basis en er geen vergoeding tegenover staat.
- …mag zonder opgaaf van reden zich op ieder moment terugtrekken van het onderzoek.

De onderzoeker...
- …verklaart de verstrekte gegevens van de hond en de eigenaar alleen ten behoeve van het onderzoek te gebruiken en zal hier vertrouwelijk mee omgaan.
- …zal het bloedmonster bij de hond zorgvuldig en met de hulp van een (poli-)assistente afnemen op de kliniek van gezelschapsdieren te Utrecht en het bloed alleen gebruiken ten behoeve van bovengenoemd onderzoek.

Gegevens eigenaar
Naam:
Adres:
Telefoonnummer:
E-mail:

Gegevens hond
Naam:
Leeftijd:
Ras:
Geslacht:
Evt. Vetware nummer

Datum en plaats: ……

Handtekening onderzoeker

Handtekening eigenaar

…… …….……..

Naam:…… Naam:……

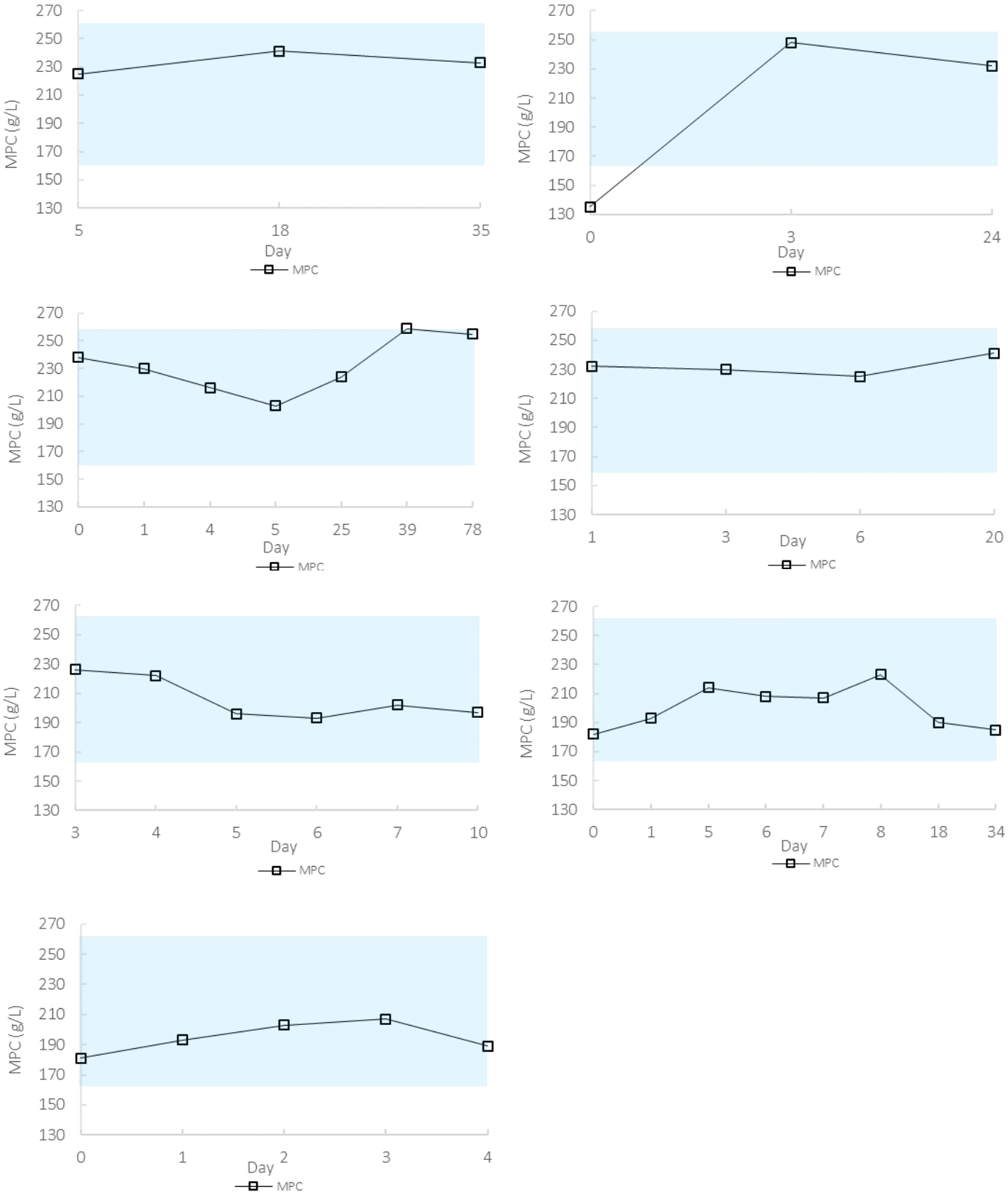


Fig. 14 Line graphs of the development of the MPC (g/L) during time (hospitalization/control appointment day). Only the cIIMHA dogs are shown that were followed a longer period of time and with multiple data points. In all patients, except one, the MPC remains within its reference range (blue box: 162 – 161 g/L). MPC: —□—.

C: Sequencing data

1. Tissue factor primer

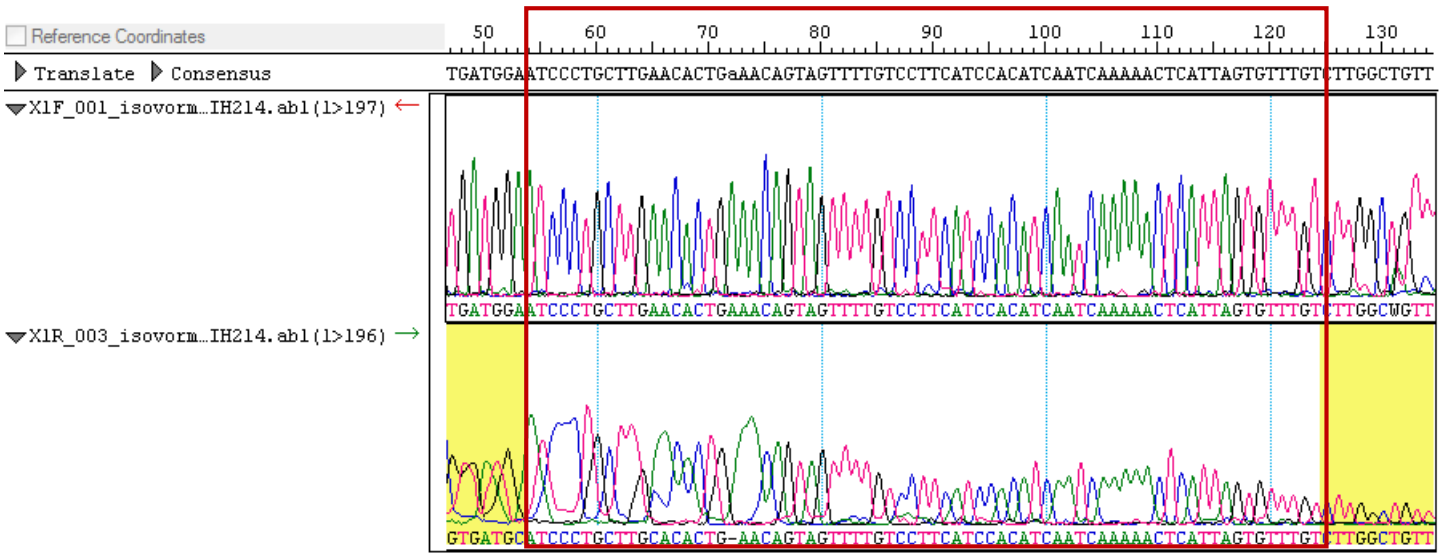


Fig. 15 Data output of the Sanger sequencing. This figure shows the presence of an identical piece of cDNA of 71 base pairs long (between the red box) in the mammary tumour sample. At this point you do not know for sure that this is encoding for tissue factor. Therefore, the next step is to analyse this piece of cDNA by use of the online programme called Ensembl (http://www.ensembl.org/index.html).

2. alternatively spliced tissue factor primer

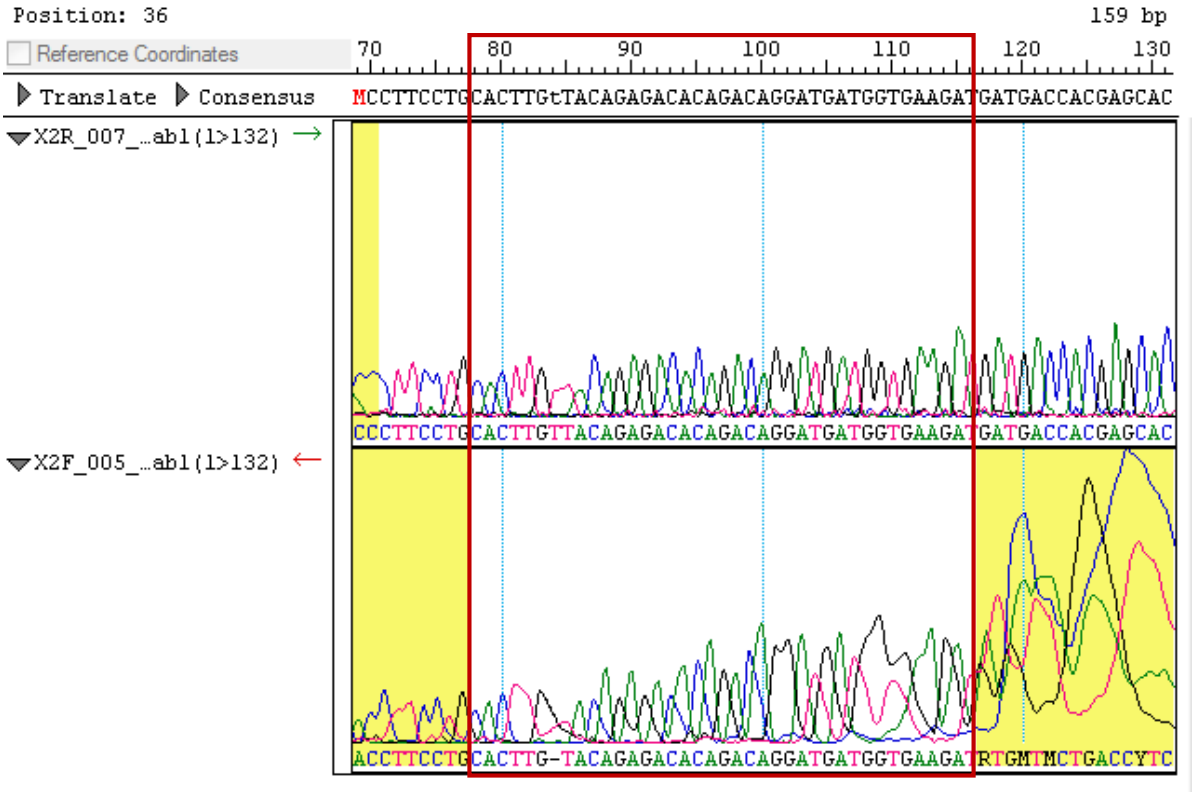
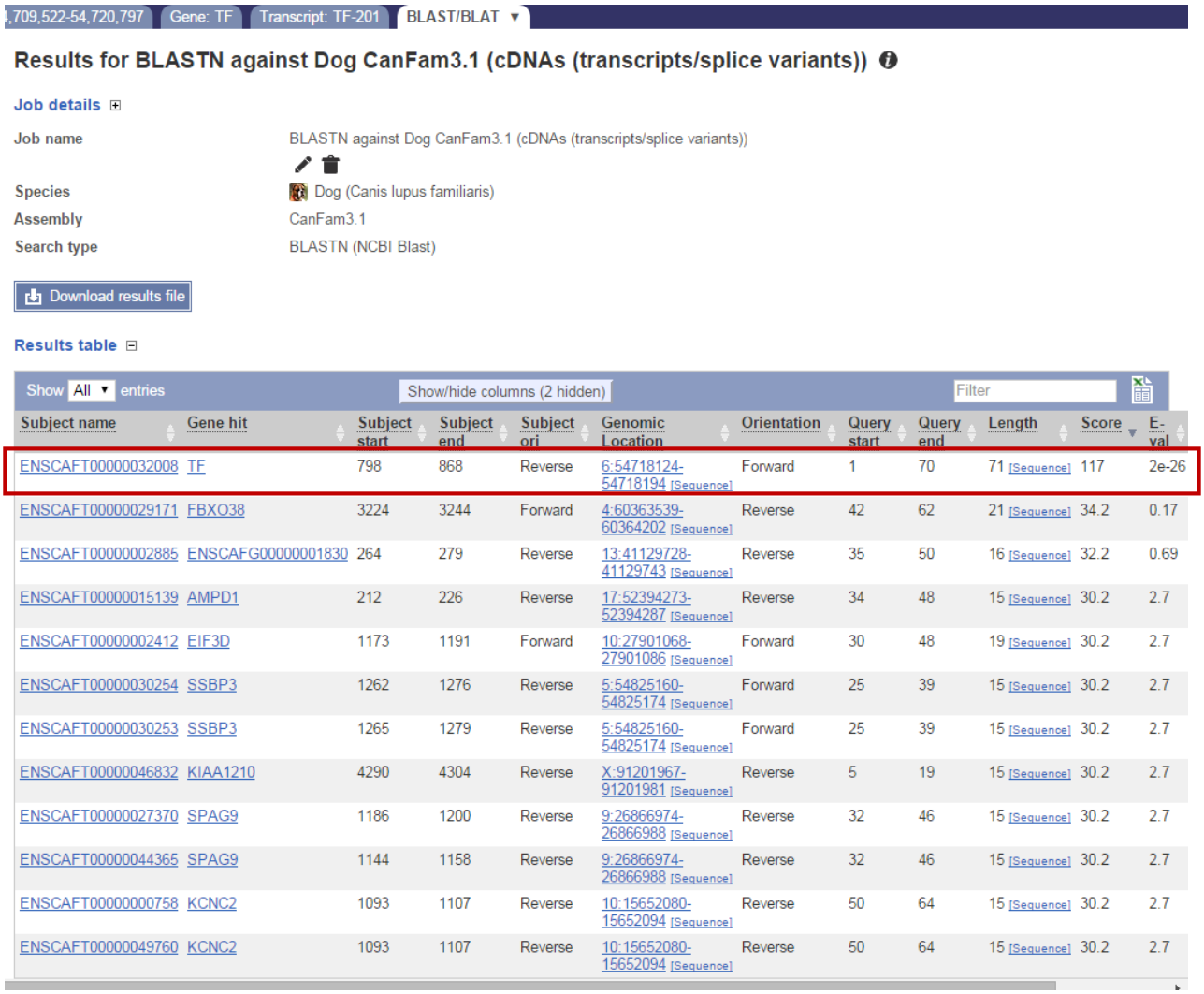


Fig. 16 Data output of the Sanger sequencing. This figure shows the presence of an identical piece of cDNA of 38 base pairs (red box) in the mammary tumour sample. At this point you do not exactly know where this piece of cDNA is coding for. So, the next step is to analyse this piece of cDNA by use of the online programme Ensembl (http://www.ensembl.org/index.html).

D: Interpretation of the sequence data

1. Tissue factor primer



,709,522-54,720,797 | Gene: TF | Transcript: TF-201 | BLAST/BLAT

Results for BLASTN against Dog CanFam3.1 (cDNAs (transcripts/splice variants))

Job details

Job name	BLASTN against Dog CanFam3.1 (cDNAs (transcripts/splice variants))
Species	Dog (Canis lupus familiaris)
Assembly	CanFam3.1
Search type	BLASTN (NCBI Blast)

Download results file

Results table

Show All entries | Show/hide columns (2 hidden) | Filter

Subject name	Gene hit	Subject start	Subject end	Subject ori	Genomic Location	Orientation	Query start	Query end	Length	Score	E-val
ENSCAFT00000032008	TF	798	868	Reverse	6:54718124-54718194 [Sequence]	Forward	1	70	71 [Sequence]	117	2e-26
ENSCAFT00000029171	FBXO38	3224	3244	Forward	4:60363539-60364202 [Sequence]	Reverse	42	62	21 [Sequence]	34.2	0.17
ENSCAFT00000002885	ENSCAFG00000001830	264	279	Reverse	13:41129728-41129743 [Sequence]	Reverse	35	50	16 [Sequence]	32.2	0.69
ENSCAFT00000015139	AMPD1	212	226	Reverse	17:52394273-52394287 [Sequence]	Reverse	34	48	15 [Sequence]	30.2	2.7
ENSCAFT00000002412	EIF3D	1173	1191	Forward	10:27901068-27901086 [Sequence]	Forward	30	48	19 [Sequence]	30.2	2.7
ENSCAFT00000030254	SSBP3	1262	1276	Reverse	5:54825160-54825174 [Sequence]	Forward	25	39	15 [Sequence]	30.2	2.7
ENSCAFT00000030253	SSBP3	1265	1279	Reverse	5:54825160-54825174 [Sequence]	Forward	25	39	15 [Sequence]	30.2	2.7
ENSCAFT00000046832	KIAA1210	4290	4304	Reverse	X:91201967-91201981 [Sequence]	Reverse	5	19	15 [Sequence]	30.2	2.7
ENSCAFT00000027370	SPAG9	1186	1200	Reverse	9:26866974-26866988 [Sequence]	Reverse	32	46	15 [Sequence]	30.2	2.7
ENSCAFT00000044365	SPAG9	1144	1158	Reverse	9:26866974-26866988 [Sequence]	Reverse	32	46	15 [Sequence]	30.2	2.7
ENSCAFT00000000758	KCNC2	1093	1107	Reverse	10:15652080-15652094 [Sequence]	Reverse	50	64	15 [Sequence]	30.2	2.7
ENSCAFT00000049760	KCNC2	1093	1107	Reverse	10:15652080-15652094 [Sequence]	Reverse	50	64	15 [Sequence]	30.2	2.7

Fig. 17 In Ensembl the whole cDNA code (71 base pairs) is very specific for canine TF. This is concluded because of the presence of the combination of the following: TF is on the first place of the list, the E-value is very low: 2e-26 and the whole sequence matches with T.

2. Alternatively spliced tissue factor primer

709,522-54,720,797 | Gene: TF | Transcript: TF-201 | BLAST/BLAT ▾

Results for BLASTN against Dog CanFam3.1 (cDNAs (transcripts/splice variants))

Job details

Job name	BLASTN against Dog CanFam3.1 (cDNAs (transcripts/splice variants))
Species	Dog (Canis lupus familiaris)
Assembly	CanFam3.1
Search type	BLASTN (NCBI Blast)

Download results file

Results table

Show All entries | Show/hide columns (2 hidden) | Filter

Subject name	Gene hit	Subject start	Subject end	Subject ori	Genomic Location	Orientation	Query start	Query end	Length	Score	E-val	%ID
ENSCAFT00000032008	TF	987	1024	Reverse	6:54719649-54719686 [Sequence]	Forward	1	38	38 [Sequence]	75.8	2e-14	100.00 [Alignment]
ENSCAFT00000009477	NUP98	7005	7021	Forward	21:26231184-26231200 [Sequence]	Reverse	9	25	17 [Sequence]	34.2	0.074	100.00 [Alignment]
ENSCAFT00000043838	ENSCAFG00000028814	3113	3128	Reverse	26:28562974-28562989 [Sequence]	Reverse	9	24	16 [Sequence]	32.2	0.29	100.00 [Alignment]
ENSCAFT00000019026	EPB42	256	271	Reverse	30:10083520-10083535 [Sequence]	Reverse	21	36	16 [Sequence]	32.2	0.29	100.00 [Alignment]
ENSCAFT00000005605	ING3	3627	3642	Reverse	14:59095312-59095327 [Sequence]	Forward	8	23	16 [Sequence]	32.2	0.29	100.00 [Alignment]
ENSCAFT00000007728	ENSCAFG00000004800	400	415	Forward	22:9483229-9483244 [Sequence]	Reverse	9	24	16 [Sequence]	32.2	0.29	100.00 [Alignment]
ENSCAFT00000045295	FUT2	3831	3846	Forward	1:107614479-107614494 [Sequence]	Reverse	8	23	16 [Sequence]	32.2	0.29	100.00 [Alignment]
ENSCAFT00000002559	TAF8	952	971	Forward	12:10709551-10709570 [Sequence]	Forward	3	22	20 [Sequence]	32.2	0.29	95.00 [Alignment]
ENSCAFT00000017140	MANBA	152	167	Reverse	32:24167861-24167876 [Sequence]	Reverse	16	31	16 [Sequence]	32.2	0.29	100.00 [Alignment]
ENSCAFT00000046337	ZNF45	1135	1149	Reverse	1:111222429-111222443 [Sequence]	Reverse	24	38	15 [Sequence]	30.2	1.2	100.00 [Alignment]
ENSCAFT00000049566	MATK	1649	1667	Reverse	20:55704219-55704237 [Sequence]	Forward	4	22	19 [Sequence]	30.2	1.2	94.74 [Alignment]
ENSCAFT00000046096	ERICH2	817	831	Forward	36:15310820-15310834 [Sequence]	Forward	24	38	15 [Sequence]	30.2	1.2	100.00 [Alignment]

Fig. 18 The Ensembl result shows that 38 base pairs of this piece of cDNA is very specific to asTF. Although it is called TF here, this piece of cDNA encodes asTF (Ensemble does not have a different subject name for the spliced tissue factor variant). The specificity is based on the first place in the list, the low E-value (2e-14) and the facts that the total length (38 base pairs) matches.

E: qPCR graphs

1. TF primer (T_A 61.4 ^oC)

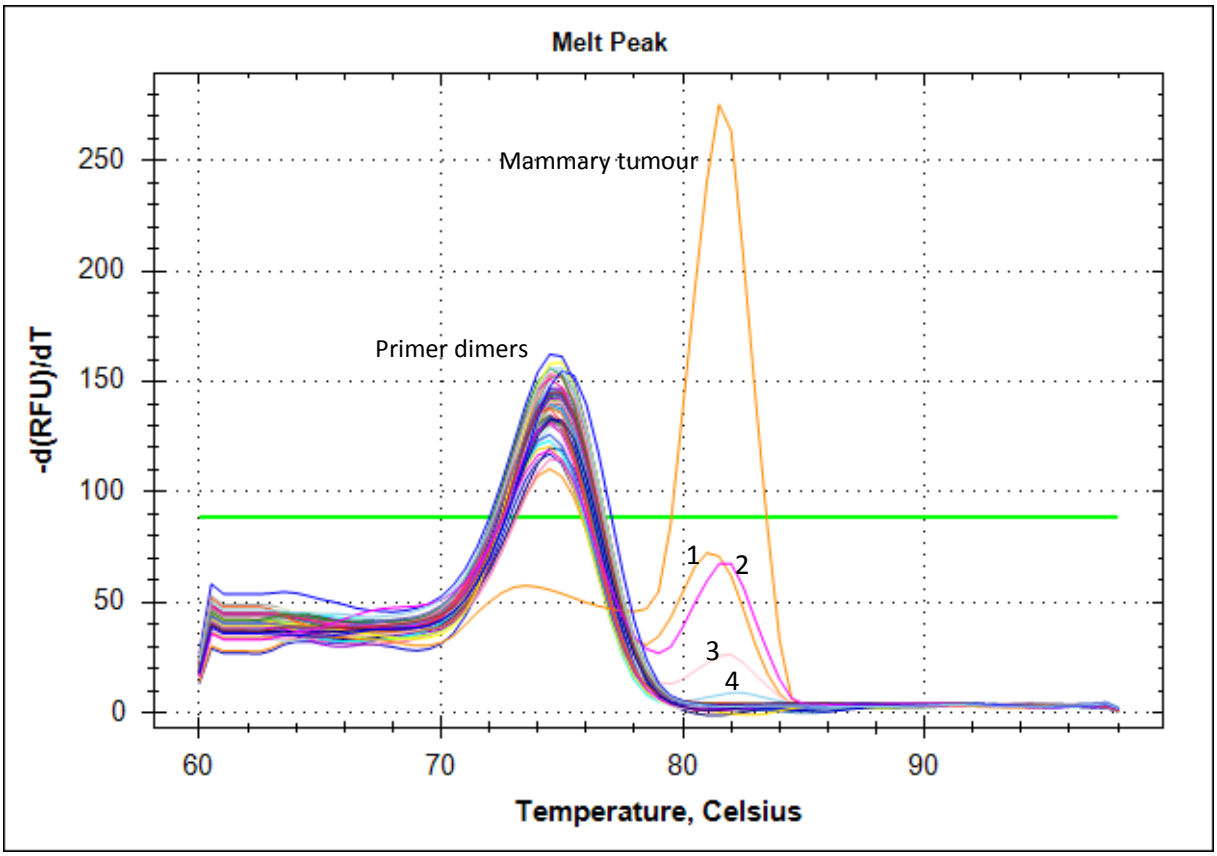


Fig. 19 qPCR plate: IMHA patients during time (all samples, n=45); healthy controls (n=11), positive control mammary tumour (n=1). The melting curve of the TF primer shows a melt peak for mammary tumour and also the same melt peaks for 4 IMHA patients (numbers 1 – 4), although the melt peaks are less high. Indicating that there is less TF mRNA present in the platelet samples. The high amount primer dimers is probably caused by the fact that there is less TF mRNA present in the platelet samples and therefore less template for the primer to bind to. So, they bind to each other and form primer dimers.

2: TF primer (T_A 61.4 ^oC)

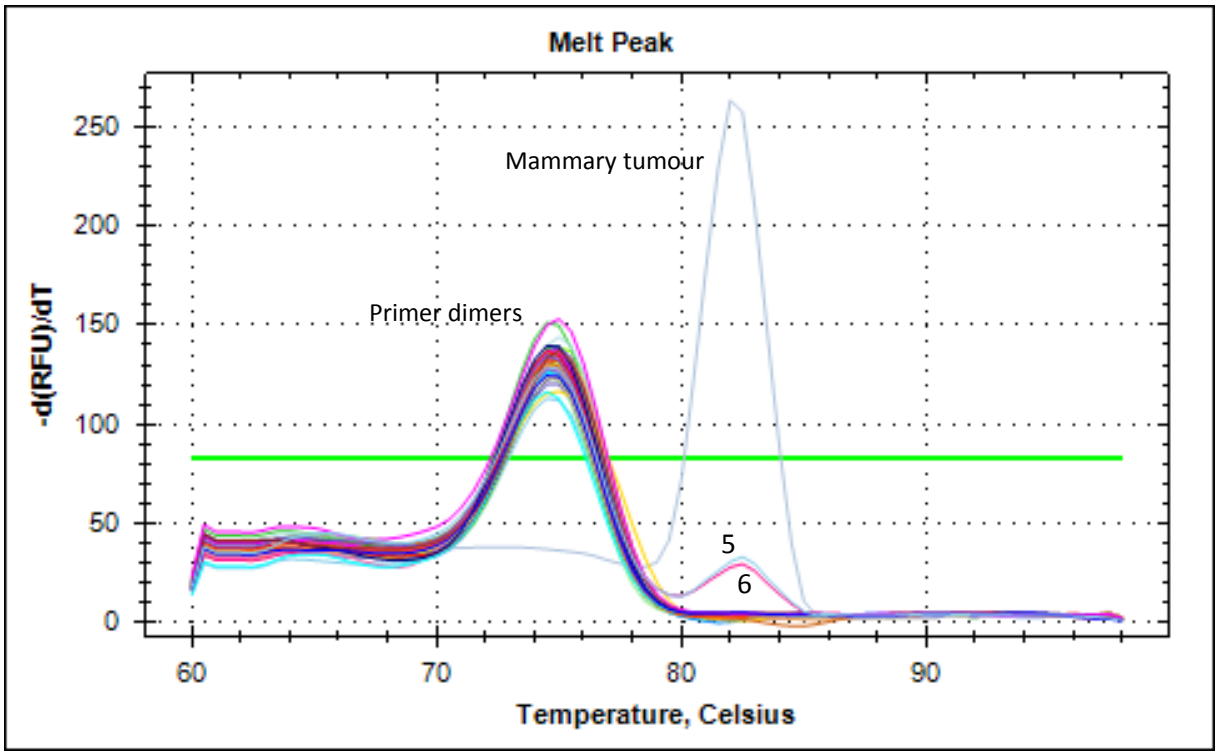


Fig. 20 qPCR plate: IMHA patients (first sample, n=12) versus other 4 groups (sepsis (n=5), DIC (n=5), tumour (n=5) and surgery (n=3)); healthy controls (n=10), positive control mammary tumour (n=1). The melting curve of the TF primer shows a melt peak for mammary tumour and also the same melt peaks for a IMHA patient (5) and a tumour patient (6), although the peaks are less high. Indicating that there is less TF mRNA present in platelet samples.