**Theses of PhD dissertation**

# GENERATION AND CHARACTERIZATION OF D-DIMER-SPECIFIC MONOCLONAL ANTIBODIES AND STUDY OF THEIR DIAGNOSTIC APPLICATION

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# 1. Introduction and aims of the study

D-dimer is a fibrin degradation product which appears in the blood as a consequence of blood coagulation, during secondary fibrinolysis. Its concentration increases as a result of elevated thrombotic activity, exact value of the plasma D-dimer level can be determined by diagnostic tests.

In human clinical practice, the D-dimer test is mostly used for the exclusion of venous thromboembolic (VTE) diseases, e.g. deep vein thrombosis (DVT) and pulmonary embolism (PE). Negative test results can exclude both diseases with high certainty in patients with low pretest probability of VTE. Apart from that, the determination of the D-dimer level plays an important role in setting up the diagnosis of disseminated intravasular coagulation (DIC) and in the monitoring of the disease.

Determination of D-dimer value is important for veterinary medicine as well since hypercoagulation results in increased D-dimer level in animals too. The diagnostic value of this phenomenon has already been investigated in several studies mainly focusing on dogs, but horses and cats have been involved in some of these researches too.

In the case of dogs, the significance of D-dimer level determination lies primarily in the diagnosis of pulmonary embolism and disseminated intravascular coagulation (DIC), but its levels may also be elevated in other diseases like thromboembolic diseases, bleeding, kidney-, heart- and liver deficiencies. Postoperative conditions and different types of cancers may also result in increased D-dimer levels.

Currently, several commercially available diagnostic tests are in use to determine D-dimer levels; however, it is a general problem that the characteristics of the tests may be quite different from each other. It is not uncommon that the different tests identify D-dimer and other degradation products in the samples to differing degrees, which results in cross-reactions and thus the specificities of these tests may be distinct from each other too. As a consequence, the different test results are not fully comparable and interfere with clinical decision-making. For all these reasons, we assumed it was imperative to develop such antibodies and diagnostic tests that were suitable for identifying D-dimer protein in the samples with due specificity.

1. The primary aim of the doctoral studies was to generate a new D-dimer-specific monoclonal antibody we aimed to use in immunoturbidimetric assays based on latex agglutination.

2. For this, our goal was performing the most comprehensive immunological characterization of the new antibody with a special focus on the cross-reactions as significant determinant of the specificity of the antibody and identification of the epitope on the D-dimer antigen.

3. Our further aim was to develop an immunoturbidimetric D-dimer diagnostic assay based on latex agglutionation, during which the antibody would be bound to the surface of latex microspheres.

4. Our ultimate goal was to study the practical application of the antibody both in human and veterinary medicines through the diagnostic test based on it.

## 2. Materials and Methods

***Generation of Monoclonal Antibodies***

Monoclonal antibodies were generated using hybridoma technology. The D-dimer antigen was prepared through the digestion of fibrin clot by plasmin. The isolated degradation product was analysed with sodium dodecil sulphate polyacrylamide gel electroforesis (SDS-PAGE) and its molecular weight was determined with Western blotting. The functional activity was measured by ELISA method (Asserachrome D-dimer, Diagnostica Stago, Inc.) and by D-dimer latex immunoturbidimetric assay (DiaSys Diagnostic Systems GmbH).

For the immunization, six 8-week-old female Balb/c AnN Crl BR mice (Charles River), were used. All animals were handled in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Veterinary Diagnostic Directorate of the National Food Chain Safety Office. The animal use protocol was approved by the Institutional Animal Care and Use Committee.

Three 8-week-old female Balb/c mice were immunized intraperitoneally (i.p.) and three others subcutaneously (s.c.) with a mixture of D-dimer antigen and complete Freund's adjuvant (CFA) (Sigma Aldrich Co.). After 24 days, the mice were reinjected in the same manner (i.p. or s.c.), using incomplete Freund's adjuvant (IFA) (Sigma Aldrich Co.). Ten days after the second injection, blood samples were collected from the caudal veins of each mouse to define the level of produced antibody with a self-developed indirect ELISA method.

Four weeks atfer the last immunization, one mouse with the highest antibody response was inoculated intravenously (i.v.) with a suspension of the D-dimer antigen and physiological saline in 1:2 dilution.

Three days later the spleen of the mouse was removed aseptically, and the spleen cells were washed in serum-free RPMI-1640 medium (Sigma Aldrich Co.). Afterwards, fusion of spleen cells with Sp2/0-Ag14 murine myeloma cells was performed in the presence of polyethylene glycol (Sigma Aldrich Co.). The fused cells were measured into 96-well microtiter plates and were allowed to grow in a carbon dioxid gas incubator, containing 5% $CO_2$ at 37°C.

The obtained hybrid cells were selected with hypoxanthine, aminopterine, and thymidine (HAT) medium (Sigma-Aldrich Co.). One week later, the HAT medium was substitued for a hypoxanthine, thymidine (HT) medium. Two weeks after the fusion, the supernatants of the grown cells were tested by the indirect ELISA method in order to identify the hybridomas producing D-dimer-specific antibodies. The cell groups selected on the basis of the ELISA testing were cloned 2 to 5 times by endpoint dilution method.

***Characterization of the antibodies***

The isotype based on the heavy and light chains of the produced antibodies was determined using a Mouse Monoclonal Antibody Isotyping Test Kit (AbDSerotec).

The possible cross-reactions of the produced antibodies were examined by the self-developed indirect ELISA method. Apart from the D-dimer antigen, different fibrin degradation products, i.e. fibrin-D monomer, fibrin-E monomer, fibrin-X fragment, fibrin-Y fragment, and also fibrinogen degradation products, i.e. fibrinogen-D monomer, fibrinogen-E monomer (all from BIOTREND Chemikalien GmbH) and fibrinogen (Sigma Aldrich Co.) were used as antigens. Analysing the results, magnitude of the examined cross-reactions was calculated from the ratio of the measured optical densities: $OD_{\text{cell supernatant}} / OD_{\text{negative control}}$.

In order to identify the epitope of the antigen, digested antigen fragments were produced. For this purpose, the D-dimer protein was dissolved in 70% formic acid and was incubated with cyanogen bromide (CNBr) (all from Sigma Aldrich Co.). The CNBr derived fragments were digested further by chymotrypsin (Sigma Aldrich Co.). The digested protein antigen fragments were separated by gel electrophoresis, their molecular weight was determined and were tested by Western blot using the generated monoclonal antibodies. After identifying the antigen fragments reacted with the D-dimer-specific antibody, the bands were cut out from

the membrane and were sequenced based on Edman degradation protocol by ABI 494 protein sequencer (Applied Biosystems).

***Cloning and Purification of Monoclonal Antibodies***

Continuous production of monoclonal antibodies was performed using different systems. One of them was a miniPERM® bioreactor (Sarstedt). During this process, the hybridoma cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, containing Antibiotic and Antimycotic solution (Sigma-Aldrich Co). The other applied system was a FiberCell® hollow fiber bioreactor, which resulted in more concentrated antibody production.

The collected cell supernatants containing the antibodies were purified by Protein G Sepharose® 4 Fast Flow (GE Healthcare) column, which works on the principles of affinity chromatography. If higher amounts of supernatants were present, ÄKTA Pure 150 (GE Healthcare) liquid chromatography system was used with 50/40 HiScale MabSelect SuRe™ (GE Healthcare) column.

The supernatans were filtered by depth filter (Sartorius stedim Biotech) to completely remove all cell debris. Afterwards, they were incubated with 1mM of protease inhibitor phenylmethylsulfonyl fluoride (PMSF) dissolved in isopropanol. Tangential filtration (Merck Millipore) was used for the concentration of the purified antibodies.

***Application of the Monoclonal Antibodies in Diagnostic Tests***

The selected monoclonal antibody was incorporated into an immunoturbidimetric assay based on latex agglutination. During this process the carboxile groups on the surface of the latex microspheres were activated and the monoclonal antibody was bound to their surface.

Measurement with the latex-reagent is based on the principle of immunoturbidimetry. During the reaction the antibodies on the surface of the latex microspheres react with the D-dimer of the samples, which has several binding sites due to its dimer properties. This results in the agglutination of the latex microspheres. As a consequence, the turbidity of the reaction medium changes and the change of the optical density between the starting and endpoint of the measuerement can be detected. Its rate is proportional to the original D-dimer concentration.

***Use of Dia-D-DIMER Diagnostic Test in Human Clinical Practice***

At the laboratory of the Department of Laboratory Medicine of the Semmelweis University, plasma samples of 158 patients suspected of venous thromboembolism (VTE) were analysed by three immunoturbidimetric assays. One of them was the assay based on the monoclonal antibody developed by our group (Dia-D-DIMER, Diagon Kft.). The other two tests were commercially available D-dimer tests: INNOVANCE® D-Dimer (Siemens AG) and STA®-Liatest® D-Di (Diagnostica Stago). Our aim was to examine the correlation between the tests and compare the results between the same samples and to align the sensitivity and specificity of the tests by estimating their optimal cut-off values.

During the evaluation, positive and negative patient groups were created from the measured data based on various cut-off ranges. Comparison of the groups was carried out using the Chi-squared test. Differences were considered to be significant at $p<0.05$ values. In the case of STA®-Liatest® D-Di and the Dia-D-DIMER tests, specificity and sensitivity values calculated at different cut-off values were represented on a ROC curve. “True positive” and “true negative” values required for the evaluation were given based on the results of the INNOVANCE® D-dimer test.

***Application of Dia-D-DIMER Test in Veterinary Practice***

The Dia-D-DIMER test was applied for the D-dimer analysis of animal samples too. Altogether seventy plasma samples were collected from dogs at two locations in Budapest with the help of the Veterinary Hematology and Oncology Center and the Animal Health Center of Budafok (Budapest, Hungary). The blood samples were placed into phlebotomy tubes (VACUETTE® Greiner AG) containing sodium-citrate buffer, then the plasma was retrieved by centrifugation (2000 x g, 10 min). The mean age of the dogs was 8 years and 2 months (1.5–14 years). Gender distribution was 52% male and 48% female. Fifty of the samples were collected from dogs diagnosed with different types of tumours. Based on the histologic examination, the following tumour types were determined in the above-mentioned population: mast cell tumour (n=6), lymphoma (n=5), sarcoma (n=10), carcinoma (n=17), insulinoma (n=3), lipoma (n=3), adenoma (n=3) and other tumour types (n=3). The control group consisted of 20 dogs, judged to be clinically healthy; they were constantly checked by a veterinary surgeon, regularly vaccinated and treated by antiparasitic medicines. The canine plasma samples were examined with Dia-D-DIMER diagnostic test, the measurements were performed on Coag XL coagulometer (Diagon Ltd.), and the D-dimer concentration was determined.

The reference range applicable for dogs was set up based on the samples of dogs judged to be healthy. The differences between certain groups (tumour-bearing dogs, and certain tumour types compared to the control group) were analysed by non-parametric Wilcoxon-Mann-Whitney test. Differences were considered to be significant at $p<0.05$ values. Statistical calculations were carried out using Minitab 14.0 statistical software (Minitab Inc.).

## 3. Results

### *Generation of Monoclonal Antibodies*

The D-dimer antigen prepared through fibrin digestion was found to be functionally active and its concentration resulted 2 mg/ml. Based on the band identified by Western blot analysis, the molecular weight of the antigen was ~180 kDa. The prepared D-dimer antigen was found to be suitable for immunization.

Altogether six Balb/c mice were immunized with the D-dimer antigen, and the spleen cells of one chosen mouse was fused with murine myeloma cells. The fused cells were selected in specific medium and 576 hybridoma cell groups were received. Using indirect ELISA method, 38 hybridoma cell groups were found to be D-dimer-positive. Based on the high titer of the produced antibody, the following hybridoma cell groups were selected: 1B4, 1H12, 2B9, 2F2, 3B3, 4G8, 6B9. These cell groups were cloned using limited dilution procedure.

### *Characterization of Antibodies*

According to the isotype determination of antibodies, 2F2 had IgM isotype. All other tested antibodies – 1B4, 1H12, 2B9, 3B3, 4G8 and 6B9 – had IgG isotype and belonged to the IgG1 subclass based on their heavy chains and to $\kappa$ isotype based on their light chains.

During the examination of the cross-reactions, we found that all antibodies reacted with the D-dimer, but the strongest reactions were shown by 1B4, 1H12, 2B9 and 3B3 antibodies. These mAbs reacted also with fibrin D and fibrinogen D fragments but did not cross-react with fibrin-E and fibrinogen-E fragments. The antibodies produced by cell lines 4G8 and 6B9 also reacted strongly with D-dimer but gave no or just weak cross-reaction with D-fragments. The weakest D-dimer reaction was given by 2F2, which reacted weakly with fibrin D, X and Y fragments as well but showed moderate reaction with fibrinogen. The cross-reaction with fibrinogen was mostly strong, moderate (3B3, 1B4, 1H12, 2F2) or weak (4G8, 6B9); however, in the case of the mAb 2B9, there was no reaction with fibrinogen at all. Due to this

remarkable trait, the cell line was selected and cloned several times. We found that the cloned cell lines also retained this trait. The antibodies produced by the clones 2B9/F2, 2B9/B1 and 2B9/D5 proved to be outstanding as the intensity of their reactions with D-dimer was very strong.

The results revealed that the cross-reactions of the 2B9 antibody and the reference Anti-D-dimer monoclonal antibody (HyTest Ltd.) were similar. Both of them reacted strongly with D-dimer but gave no cross-reaction with fibrinogen and reacted weakly with fibrin X and Y fragments. Moreover, the clones indicated above reacted more strongly with D-dimer and weaklier with D fragments in comparison to the reference antibody.

Antibody produced by the clone 2B9/D5 was used for the determination of the D-dimer epitope. The reduced and unreduced forms of the antigen fragments digested by chymotrypsin were separated by gel electrophoresis and then tested by Western blot analysis. As a result of the Western blotting, bands representing 25, 30 and 38 kDa fragments of the unreduced sample showed antigen-antibody reactions. The fragments identified with molecular weight of 25 and 38 kDa provided sequences, whereas no sequences were obtained from the 30 kDa fragment. The identified amino acid sequences at chain B of fibrin D monomer were 94 IQPDSS- and 140 GNVANTNT-, and 23 LQEIYNSNNQ- and 93 VYCEID- at chain C. The structural analysis of the fragments revealed a spatial epitope structure on the surface of the D-dimer molecule instead of a simple sequential epitope.

The identified epitope surfaces are located relatively distant on the fibrin subunits; therefore, steric interferences have no or low probability between the two reacting antibodies bound to the epitope. This suggests that antibody 2B9 could be an appropriate reporter molecule in latex immunoassays.

***Diagnostic Application of the 2B9 Monoclonal Antibody***

The 2B9 monoclonal antibody developed by our group was used in an immunoturbidimetric diagnostic assay based on latex agglutination. The test is marketed under the name of Dia-D-DIMER and was developed at Diagon Ltd.

***Measurement of D-dimer Concentration in Human Samples***

Dia-D-DIMER and STA®-Liatest® D-Di tests were compared to INNOVANCE® D-Dimer test during the analysis of the human plasma samples. Based on Bland-Altman analysis of STA®

-Liatest® D-Di and INNOVANCE® D-Dimer tests, the mean deviation was 0.43, standard deviation was 1.33 and the maximum deviation from the mean was 11.64. Comparison of INNOVANCE® D-Dimer and Dia-D-DIMER tests resulted in the mean deviation of 0.47, standard deviation of 1.92 and maximum deviation from the mean of 13.5. Regression analysis of the tests gave the following $R^2$ values: $R^2$=0.8973 in the case of INNOVANCE® D-Dimer and STA®-Liatest® D-di, and $R^2$=0.7357 in the case of INNOVANCE® D-Dimer and Dia-D-DIMER.

Positive and negative patient groups were created from the concentration results given by the three different D-dimer tests. Comparison of the groups were carried out using the Chi-squared test. Although the cut-off values of STA®-Liatest® D-Di test were altered between 0.3 and 1 µg/ml FEU, no significant difference was found in comparison to INNOVANCE® D-Dimer test ($p>0.05$). In the case of Dia-D-DIMER, the two tests showed significant difference between 0,2-0,3 µg/ml FEU cut-off values ($p<0,05$). Borderline difference ($p=0.048$) was observed at 0,4 µg/ml FEU, and the tests clearly showed no difference between 0.5-1 µg/ml FEU.

Based on the different cut-off values, specificity and sensitivity values of Dia-D-DIMER and STA®-Liatest® D-Di tests were determined. INNOVANCE® D-Dimer test was used as reference test. Sensitivity values of STA®-Liatest® D-Di were varying between 82.7-100% in the case of 0.2-1 µg/ml FEU cut-off values, while those of Dia-D-DIMER were found between 92.3-100%. Specificity values of the tests were between 50-96.3% and 35.2-87%, respectively. Based on this data, receiver operating characteristic (ROC) curve of the two tests was created. The area under the curve (AUD) represented the efficiency of the test. In the case of STA®-Liatest® D-Di test, this value was 0.9804, while for Dia-D-DIMER, it was 0.9707.

Optimum cut-off value of the tests can be estimated by the value of the highest accuracy of the tests (0.962 and 0.918) and by the highest value of the Youden Index (0.925 and 0.804). For STA®-Liatest® D-Di, it corresponded to 0.5-0.6, while in the case of Dia-D-DIMER, it was 0.7 µg/ml FEU.

***Measurement of D-dimer Concentration in Canine Samples***

Plasma samples of 20 clinically healthy and 50 tumour-bearing dogs were measured by Dia-D-DIMER test.

For benign tumours we did not find significant deviation in comparison to the control group (median/0.34 µg/ml [FEU]; p=0.1310). However, in the case of malignant tumours, significantly elevated concentrations were found compared to the group of healthy dogs (median=0.68 µg/ml [FEU]; p=0.0002).

The median of D-dimer levels in samples of dogs with metastatic cancer was 1.01 µg/ml FEU, which is higher than that of the overall malignant group. Significant difference was found between the metatstaic and the control group (p=0.0016).

The samples were grouped according to the histological grades and stages of tumours as well. In the case of Grade I, the median was 0.40 µg/ml FEU, while for Grades II-III, higher median was found (0.94 µg/ml [FEU]). Similar conclusion was reached by the examination of tumour stages since the medians were far higher in the case of higher stages – for Grades IV-V – (1.50 µg/ml [FEU]) than in the lower ones – for Grades I-II-III (0.44 µg/ml [FEU]).

The neoplastic groups were analysed based on tumour types too. Compared to the control group, the highest D-dimer median was calculated from the group of dogs with malignant lymphoma (1.72 µg/ml FEU). Likewise, significantly high concentration value – 0.83 µg/ml FEU – was occurred in the animal group suffering from carcinoma too. In the case of the group with lipoma and insulinoma, the medians were low, similar to the control group. However, in these groups, the sample size was low too. Based on the Mann-Whitney statistical test, the mast cell tumour group (p=0.1024) did not differ significantly from the control group. On the other hand, the lymphoma (p=0.0114), sarcoma (p=0.0005) and carcinoma (p=0.0022) groups displayed significant difference.

Reference range for the analysis of neoplastic canine plasma samples was set up (0.06-0.69 µg/ml [FEU]) based on the samples of healthy dogs. Positive and negative groups were created from the measured data based on the cut-off value.

With altered cut-off values, sensitivity, specificity and positive and negative predictive values (PPV and NPV) of the Dia-D-DIMER test were calculated for canine tumours. According to our results, with cut-off values ranging from 0.3 to 0.69 µg/ml FEU, the sensitivity values were found between 33 and 51%, while those of the specificity were between 85 and 100%.

By applying the upper limit of the reference range (0.69 µg/ml [FEU]), we reached 33% sensitivity and 100% specificity. For the samples of tumour-bearing dogs, the positive predictive value is 100%, while the negative predictive value is 29%. Therefore, in the case

of the Dia-D-DIMER test, samples with concentrations lower than 0.69 µg/ml FEU cut-off values were considered as negative in terms of malignancy, while results above this value were considered as positive.

# 4. Discussion

***Characteristics of the Generated Antibodies***

The generated, new monoclonal antibody 2B9 meets the criteria established by the reaction mechanism of immunoturbidimetric D-dimer test as follows.

***Study of Cross-Reactions***

Based on the results of cross-reaction analysis, we experienced that antibody 2B9 recognises the D-dimer antigen with appropriate specificity and does not react with fibrinogen, which is an important characteristic of an antibody intended to be used in D-dimer diagnostic tests. This advantageous characteristic makes the novel antibody suitable for assays measuring citrated plasma or even whole blood samples, since studies about D-dimer test harmonization suggest that anti-D-dimer antibodies should not react with fibrinogen or fibrinogen degradation products, nor with such fibrin and fibrinogen fragments that were produced during proteolysis induced by other enzymes.

We also considered it as a positive outcome that according to the results of the cross-reaction analysis, antibody 2B9 showed quite similar properties to the reference HyTest Anti-D-dimer monoclonal antibody, which is produced by a renowned company (HyTest Ltd.).

***Evaluation of the Epitope Mapping***

Based on the epitope mapping, we can conclude that antibody 2B9 recognises epitopes located symmetrycally and relatively far from each other on the surface of the D-dimer. Their position sterically allows and facilitates the agglutination of the latex beads.

The commercially available anti-D-dimer monoclonal antibodies detect the epitopes on the surface of the D-domain of fibrin fragments crosslinked through Factor XIIIa. Each of these monoclonal antibodies has its own specificity.

Epitope sequence of the first patented anti-D-dimer monoclonal antibody (DD-3B6/22) and that of an antibody designated B4 was determined and published. Although researchers

generated several D-dimer-specific antibodies in the past few years, to the best of our knowledge, their epitope mapping cannot be found in the literature. Our epitope analysis showed that the monoclonal antibody 2B9 recognises different sequences on the surface of D-dimer like other D-dimer-specific antibodies in the literature.

***Practical Application of the Generated Antibodies***

Using the D-dimer-specific monoclonal antibody designated 2B9, the Hungarian Diagon Ltd. developed an *in vitro* diagnostic (IVD) immunoturbidimetric D-dimer assay named Dia-D-DIMER. The product has passed clinical evaluation, bears CE marking and is currently commercially available.

***Evaluation of the Dia-D-DIMER Test Results in Human Application***

The commercial test, which is based on the 2B9 D-dimer-specific monoclonal antibody, has passed the clinilcal evaluation successfully. In addition, a further study by us has also demonstrated its applicabilty in human clinical practice.

With the analysis of human plasma samples, our aim was to compare the Dia-D-DIMER test with two other, commercially available D-dimer tests. One of them was chosen as reference and we characterised the other tests in comparison to this.

Bland-Altman analysis was performed to characterise the agreement between the tests, during which both the Dia-D-DIMER and the STA®-Liatest® D-Di were compared to the reference test. The slope of the line applied to the values representing the difference and average values of the tests gave lower value for the STA®-Liatest® D-Di and the reference test than for the Dia-D-DIMER. Accordingly, in the case of higher D-dimer concentration values, we found minor difference between STA®-Liatest® D-Di and the reference.

The results of the above-mentioned two pairs of tests were compared using linear regression analysis. The $R^2$ value, representing the degree of correlation, was higher ($R^2$=0.8973) in the case of the test pair involving STA®-Liatest® D-Di and lower in the case of Dia-D-DIMER ($R^2$=0.7357), which indicates a slightly stronger correlation.

The differences between the tests may be resulted by several factors. Obviously, on user level, we already have no possibility harmonizing the tests by modifying the antibody or the construction. However, fine-tuning of the positive and negative results of the tests may be achieved by modifying the cut-off value. The data unequivocally indicate that the sensitivity

and the specificity values change considerably if the cut-off value is modified. Sensitivity increases by choosing a lower cut-off value since the number of the negative results decreases, however, in this case, the number of false positive results may grow. In contrast to this, if the cut-off value raises, higher specificity can be achieved. Also, it gives more false negative and fewer false positive results.

In an attempt to establish an optimum cut-off value, we found that the following concentration values harmonized the best with 0.5 µg/ml FEU applied for INNOVANCE® D-dimer: 0.5-0.6 µg/ml FEU for STA®-Liatest® D-Di and 0.7 µg/ml FEU for Dia-D-DIMER. Our results support the recommendations in the literature, according to which the"common" D-dimer cut-off value is usually 0.5 µg/ml FEU, however, other values may prove to be more suitable for this purpose.

***Application of the Dia-D-DIMER Test in Canine Samples***

Primarily, the Dia-D-DIMER test was developed for the measurement of D-dimer level in human plasma samples. Based on the literature – i.e. human D-dimer tests could be made suitable for the testing of animal samples too –, we attempted to determine the D-dimer level of canine plasma samples. In our studies, we found that the Dia-D-DIMER test using the 2B9 antibody is suitable for determining the D-dimer concentration of canine plasma samples as well.

***Connection between D-dimer and Tumours in Dogs***

Our results show that the D-dimer concentration measured for the group of tumour-bearing dogs was significantly higher than for the control group. These results are consistent with several other results in the literature, which are mainly based on the analyses of human samples. Unfortunately, fewer similar studies have been carried out on dogs.

Grouping the tumours based on histological types, we found significantly higher D-dimer concentrations in the group of dogs with malignant tumours (lymphoma, sarcoma and carcinoma) compared to the control group. However, in the case of dogs suffering from equally malignant mast cell tumour, no significant difference was found in comparison to the control.

During our research, we found that the presence of metastases and the aggressiveness of the tumours are also reflected in the D-dimer results. As compared to the group of healthy dogs, the analysis of the plasma samples of dogs in the malignant and metastatic tumour

groups display significantly higher D-dimer concentrations. However, in the case of dogs with benign tumours, no significant deviation could be shown. These results also support the data found in the literature.

Several recent studies have revealed that there is a correlation between plasma D-dimer levels and tumour progression (stage and grade of tumour), and it has been supported by our results too. Our research has also confirmed the correlation between higher grade or stage and the increased D-dimer value in the case of tumour-bearing dogs.

Unfortunately, in veterinary literature, we have found no publications about the measurement of D-dimer level in the case of tumour-bearing dogs with respect to specificity and sensitivity. Nevertheless, in our research, 30% sensitivity and 100% specificity were obtained by the application of a determined cut-off value (0.69 µg/ml [FEU]), while the PPV was also 100%. Due to the high specificity of the test, 100% of dogs with negative test results were de facto clinically healthy. On the other hand, due to the low sensivitiy of the test, we obtained fewer positive results for dogs diagnosed with cancer. Further research is however required to determine whether the measurement of D-dimer concentration in dogs and other animals could be an additional diagnostic tool, especially in the detection of neoplasia and in the monitoring of disease progression and effectiveness of treatment.

## 5. New scientific results

1, We generated and characterised a D-dimer-specific monoclonal antibody (2B9) that recognises a new spatial epitope on the surface of the D-dimer molecule, which has not been published in the literature yet.
2, We identified the following amino acids on chain B of fibrin D monomer: 94 IQPDSS and 140 GNVANTNT and on chain C: 23 LQEIYNSNNQ- and 93 VYCEID as parts of a spatial epitope surface.
3, The generated 2B9 antibody was incorporated into a domestically developed, new immumoturbidimetric D-dimer assay named Dia-D-DIMER, which bears CE marking and is commercially available.
4, In veterinary practice, suitability of the Dia-D-DIMER test as a diagnostic tool for thromboembolic disorders and diseases associated with certain tumors in dogs was proven. It may also play an important role in monitoring the possible clinical progression of tumors as well.

# 6. Scientific publications

***Publications in scientific journals***

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