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THROMBOELASTOGRAPHIC ANALYSIS OF HEMOSTATIC ABNORMALITIES IN DOMESTIC ANIMALS

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Thromboelastographic Analsysis of Hemostatic Abnormalities in Domestic Animals

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Thromboelastographic Analysis of Hemostatic Abnormalities in Domestic Animals

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DEDICATORIA Y AGRADECIMIENTOS

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RESUMEN

La hemostasia (del griego *hemo,* sangre y *stasis*, estabilidad) se define como la detención de la pérdida de sangre. Sin embargo, el objetivo final del sistema hemostático es mantener un equilibrio perfecto entre la hipercoagulabilidad, con tendencia a la trombosis y el extremo opuesto del espectro o hipocoagulabilidad, con tendencia a la hemorragia. A principios de los años 60 se propuso un modelo de coagulación a modo de cascada que consiste en una serie de pasos en los que un complejo proteasa-enzima activa a un zimógeno, es decir, un factor de coagulación que conduce a la activación de otro, lo que finalmente resulta en la generación de trombina que, a su vez, convierte el fibrinógeno en fibrina, acontecimiento fundamental para la formación del coágulo. Las revisiones más recientes del modelo de hemostasia en cascada enfatizaron la relevancia de las células portadoras de factor tisular (TF), ahora identificado como esencial para la formación de trombina, en un proceso de coagulación gradual que surge después de la ruptura de la integridad del endotelio vascular y que se define en los siguientes pasos:

a) Iniciación de la coagulación en células portadoras de TF.

b) Amplificación de la señal procoagulante por la trombina generada en la célula portadora de TF.

c) Propagación de la generación de trombina en la superficie de las plaquetas.

d) Fase de terminación. Responsable de limitar y localizar la generación de trombina.

Una vez que el vaso ha sido sellado, el sistema fibrinolítico se activa, convirtiendo la fibrina en sus productos de degradación solubles

a través de la acción de la plasmina. La fibrinólisis se inicia cuando los activadores de plasminógeno convierten la proenzima plasminógeno en plasmina por las proteasas asociadas a las células endoteliales vasculares. La fibrinólisis remodela el coágulo y permite un flujo normal de sangre a través del vaso afectado.

Las anomalías significativas del sistema hemostático se manifiestan en forma de sangrado y/o de trombosis. El sangrado puede ocurrir si hay una formación anormal del tapón de plaquetas y/o una generación reducida de la trombina durante la formación del coágulo de fibrina en el sitio de la lesión vascular. Son lo que denominamos trastornos de la hemostasia primaria y secundaria, respectivamente. El sangrado también puede ocurrir si el coágulo de plaquetas/fibrina se degrada prematuramente debido a la fibrinólisis excesiva, lo que conocemos como trastornos de la hemostasia terciaria. El riesgo de sangrado se estima en base al historial médico del paciente, el examen físico, el posible trastorno subyacente y los resultados de las pruebas de coagulación. La mayoría de las pruebas de coagulación comunes, incluyendo el tiempo de protrombina (PT), el tiempo parcial de tromboplastina activado (aPTT) y los niveles de fibrinógeno, nos informan acerca de los niveles en plasma de los factores de coagulación solubles requeridos para la hemostasia. Pero es obvio que las pruebas clínicas de coagulación comúnmente utilizadas no reflejan realmente la complejidad de la hemostasia *in vivo*. Las pruebas de coagulación de sangre entera tienen la ventaja de que pueden reflejar las contribuciones de las plaquetas y otras células circulantes a la hemostasia y, por lo tanto, tienen la capacidad para evaluar la interacción célula/proteína, lo que da cobertura a los principios enunciados en la teoría del modelo celular de hemostasia descrito anteriormente.

La tromboelastografía (TEG) permite un análisis *ex vivo* del sistema hemostático proporcionando información sobre la hemostasia primaria, secundaria y terciaria. En la TEG, una copa cilíndrica que contiene una muestra de sangre entera de 340 µl oscila a 4° 45' cada 5 segundos con un pin en un alambre de torsión introducido en la sangre. Cuando el coágulo se adhiere al pin, este transmite una señal eléctrica que posteriormente se representa en forma de una gráfica. Una serie de variables definen esa gráfica de la TEG, y esas variables se asocian a su

vez con el proceso de coagulación. Por ejemplo, el tiempo TEG_R se puede comparar con PT y con aPTT. Las variables TEG_K, TEG_α y TEG_{MA} se asemejan a la concentración de fibrinógeno y al recuento de plaquetas. Las variables de lisis de coágulos LY30 y LY60 pueden estar a la par con los productos de degradación de fibrinógeno y los D-dímeros.

El desarrollo inicial de la TEG data de 1940 y se sitúa en Alemania, pero fue en la década de los 80 cuando la TEG se convirtió en un foco primario de investigación debido a la necesidad de superar las limitaciones de las técnicas de análisis hemostático común para la evaluación de la hemostasia (p. ej., disfibrinogenemias adquiridas, presencia de inhibidores de la coagulación como heparina, degradación de fibrina). Los resultados de esa investigación dieron lugar a algoritmos de TEG que se utilizaron para optimizar el uso y la transfusión de productos sanguíneos a pacientes coagulopáticos y que resultaron en un menor uso de productos sanguíneos.

Las complicaciones hemorrágicas perioperatorias asociadas con procedimientos quirúrgicos específicos (p. ej., *bypass* cardíaco, trasplante de hígado) han sido descritas con detalle en seres humanos; sin embargo, existen muy pocas referencias en medicina veterinaria. Se ha demostrado que hasta el 26% de los galgos de carreras retirados (RRG) padecen hemorragias excesivas 24 a 48 h después de someterse a gonadectomías rutinarias y/o a la amputación de extremidades, hasta el punto de requerir transfusión de componentes sanguíneos. Los perfiles preoperatorios de hemostasia, el análisis de la función plaquetaria y la concentración de vWF no pueden identificar un trastorno hemostático ni predecir qué RRG sangra después de la cirugía. Identificar los perros con mayor riesgo de sangrado o simplemente desarrollar un protocolo de prevención de sangrado excesivo sería extremadamente valioso. Con dicho propósito nos propusimos analizar comparativamente la hemostasia en RRG sanos y perros no galgos sanos por medio de la TEG. Para ello se incluyeron 28 RRG y 15 perros no galgos todos sanos y sin antecedentes de trastornos hemorrágicos ni terapias que pudieran afectar la hemostasia. Los perros se consideraron sanos en función de la ausencia de signos clínicos de enfermedad,

resultados del examen físico (PE), hemograma completo (CBC) y panel de hemostasia (aPTT, PT, fibrinógeno).

Nuestros resultados revelaron notables diferencias entre perros RRG y no galgos para aPTT, hematocrito (HCT), recuento de plaquetas (PLT), TEG_K, TEG_α, TEG_{MA} y TEG_G. En RRG la cinética de la formación del coágulo fue más lenta y la fuerza coágulo más débil que en los perros no galgos. Estos resultados confirman la tendencia a sangrar observada después de un trauma menor o procedimientos quirúrgicos en RRG. El elevado HCT y bajo recuento PLT no son más que características hematológicas bien conocidas de los RRG, lo cual habla de sus propiedades reológicas únicas. Por otra parte, la alta viscosidad de la sangre entera en RRG (HCT en perros no galgos: 41,81% vs. 47,61% en RRG) podría causar una disminución en los parámetros TEG_{MA} y TEG_G debido a la restricción de movimiento del pin transductor en la copa cilíndrica del TEG. Además, como los RRG tienen menos plasma por unidad de volumen, los valores más bajos de TEG_{MA} y TEG_G podrían ser el resultado de una adición de anticoagulante excesiva. Sin embargo, de acuerdo con las Directrices del Comité Nacional de Estándares de Laboratorio Clínico de los EE. UU. para la recolección, transporte y procesamiento de muestras de sangre para analizar el ensayo de coagulación basado en plasma, la concentración de anticoagulante solo debe ajustarse en pacientes con HCT > 55%. Solo 2 RRG en este estudio tenían HCT > 55% (55.7 y 59%, respectivamente).

Una vez caracterizadas las peculiaridades hemostáticas de los RRG a través de la TEG, pudimos abordar el siguiente paso que consistió en identificar las singularidades hemostáticas perioperatorias en RRG. Se incluyeron en el estudio un total de 21 perros RRG sanos sin antecedentes de trastornos hemorrágicos ni anormalidades en PE, CBC, perfiles de bioquímica sérica (en perros de más de 5 años) y con prueba SNAP (SNAP-4DX; Laboratorios IDEXX) negativa para enfermedades transmitidas por vectores comunes. Al estudiar los trastornos hemorrágicos perioperatorios en RRG sometidos a gonadectomías no encontramos diferencias significativas entre "sangrantes" y "no sangrantes" para los parámetros preoperatorios de TEG, aPTT, concentración de fibrinógeno, recuento de PLT o concentración de

hemoglobina (HGB). Sin embargo, el incremento en los parámetros TEG_{MA} y TEG_G observados en los RRG no sangrantes sugiere la formación de un coágulo más fuerte en este grupo que se interpreta como una respuesta de apoyo a la hipercoagulabilidad fisiológica inducida por la inflamación o el estrés asociado a la cirugía. Hasta donde conocen los autores, este estudio fue pionero en documentar una fase reactiva de hipercoagulabilidad postoperatoria en perros. También es posible que, puesto que el síndrome de sangrado en RRG se debe a una interacción defectuosa de células y proteínas, las pruebas de coagulación convencional cuantitativa (p. ej., recuento de PLT) y aquellas basadas en plasma sanguíneo (p. ej., aPTT, PT, concentración fibrinógeno) no sean adecuadas para diagnosticar el síndrome de sangrado de los RRG. En nuestro estudio, la TEG fue la única prueba de diagnóstico (por los parámetros TEG_{α}, TEG_{MA} y TEG_G) que se correlacionó con los signos clínicos de hemorragia postoperatoria. Por lo tanto, la TEG puede ser una prueba útil para evaluar el riesgo perioperatorio de sangrado en RRG.

A continuación, centramos nuestra investigación en la correlación entre el cáncer y las coagulopatías. La hipercoagulabilidad en cáncer se ha relacionado con la capacidad del tumor para sobre expresar procoagulantes, como TF, a través de la interacción de las células tumorales con plaquetas, macrófagos-monocitos y células endoteliales vasculares. Este fenómeno se denomina como fenotipo de células protrombóticas. Varios estudios en humanos han demostrado una fuerte asociación entre la trombosis venosa idiopática y posterior desarrollo de cáncer clínico y la prevalencia de la trombosis, hasta un 30%, en pacientes con cáncer. Es obvio que identificar perros en estado protrombótico para establecer tratamientos que reduzcan el riesgo de trombosis puede ser de interés clínico en medicina veterinaria. Así, establecimos como siguiente objetivo analizar las características hemostáticas en perros con carcinomas utilizando la TEG y otros marcadores de coagulopatías como el PAI-1 (Inhibidor del Activador de Plasminógeno) y el TAT (complejos Trombina-Antitrombina).

En este estudio incluimos un total de 32 perros con carcinomas sin antecedentes de haber recibido terapia anticancerígena y/o inhibidores de COX (ciclooxigenasas) al menos 2 semanas antes del muestreo. Los

perros con carcinomas mostraron una generación de trombos más rápida (TEG_G 834.8 ± 91.1 vs. 707.8 29 ± 75.8 mm / min; media ± SE), aumento de la concentración de fibrinógeno (276 vs. 151 mg / dL) y recuento de PLT (425 vs. 324 x 10^9 U / L), pero una menor actividad de PAI-1 (15.7 vs. 26.2 UI/ml). Las anomalías hemostáticas más comunes encontradas en perros con carcinoma fueron: hipercoagulabilidad (TEG_G > media + 2SD de perros sanos) en 15/32 (46%), trombocitosis (PLT > 424 x 10^9 U /L) en 15/32 (46%), e hiperfibrinogenemia (fibrinógeno > 384 mg/dl) en 9/32 (28%). La hiperfibrinogenemia parece estar presente en el estado hipercoagulable en perros con carcinoma, ya que el 61% de los perros hipercoagulables con carcinoma eran hiperfibrinogenémicos. Como era de esperar, los tiempos de coagulación (PT, aPTT) fueron similares entre los perros con carcinoma y sin carcinoma, lo que respalda la falta de capacidad de los ensayos hemostáticos con plasma para detectar la hipercoagulabilidad.

Los complejos TAT se crean después de la producción de trombina y se han propuesto como un marcador subclínico de formación de trombina. Nuestros resultados, sin embargo, no demostraron diferencias entre los perros con carcinoma y sin carcinoma para la formación de los complejos TAT. PAI-1 y otros componentes del sistema fibrinolítico juegan un papel importante como moduladores de la angiogénesis y metástasis tumorales, pero aún existe controversia sobre la relevancia biológica de esta proteína en la tumorigénesis. Por ejemplo, una alta expresión de PAI-1 se asocia con un mal pronóstico en varios tipos de cáncer en humanos. Sin embargo, en ratones las concentraciones más bajas de PAI-1 aumentaron la angiogénesis hasta tres veces, pero a altas concentraciones la inhibieron. En nuestro estudio, la actividad PAI-1 en perros con carcinoma fue significativamente menor que en el grupo control, un resultado llamativo que anima a seguir investigando sobre el papel que puede jugar esta molécula en la progresión del tumor.

Profundizando en nuestras investigaciones sobre coagulopatías y cáncer, el siguiente paso fue el estudio de la CID (Coagulación Intravascular Diseminada) usando la TEG. La CID se puede describir y categorizar en dos etapas: la etapa inicial "no manifiesta" está dominada por la hipercoagulabilidad; debido al consumo continuo de los factores de coagulación y de las plaquetas que tiene lugar durante la etapa inicial,

se alcanza un nivel en el que no se puede mantener la función hemostática normal, lo que conduce a un estado de hipocoagulabilidad o etapa “manifiesta”, en la que se pueden observar signos clínicos de sangrado espontáneo en el paciente. Para este estudio empleamos un perro Bichon Frise de 10 años en el que se había establecido un diagnóstico de hemangiosarcoma visceral con metástasis pulmonar. El análisis de la hemostasia reveló un tiempo de coagulación prolongado (es decir, PT muy elevado y aPTT fuera del rango), hipofibrinogenemia y aumento de D-dímeros, por lo que en base a estos hallazgos se realizó un diagnóstico presuntivo de CID. Las gráficas de TEG se correlacionaron positivamente con los resultados de las pruebas de coagulación anteriores mostrando hipocoagulabilidad (es decir, TEG_K prolongado —cinética del coágulo—; coágulo más débil con un TEG_{MA} bajo) e hiperfibrinólisis (es decir, aumento de TEG_{LY30} y TEG_{LY60}). En base a estos resultados determinamos que el perro estaba en la fase hiperfibrinolítica de CID y, por tanto, que la TEG es una prueba diagnóstica valiosa para su diagnóstico en perros. Los parámetros fibrinolíticos que aporta la TEG (es decir, TEG_{LY30} y TEG_{LY60}) pueden agregar información importante para el diagnóstico de la CID, pero también para su tratamiento y pronóstico.

Nuestro último estudio de investigación se centró en equinos que sufren enfermedades comunes y frecuentemente asociadas con alteraciones de la hemostasia. Se ha sugerido que los depósitos de fibrina acumulados en los capilares de los órganos (p. ej., riñón, hígado, pulmón) están vinculados al estado hipercoagulable observado en la CID. La TEG podría ser extremadamente útil si sirviera para identificar con la suficiente antelación caballos en riesgo de desarrollar CID o al menos antes de que se produzcan grandes depósitos de fibrina en los capilares de los órganos, conduciendo a una insuficiencia multiorgánica. Con esa antelación sería posible establecer tratamientos basados en productos sanguíneos y también un pronóstico más ajustado a la realidad. Por lo tanto, nuestro objetivo fue investigar mediante TEG las características hemostáticas en caballos con enfermedades que suelen llevar asociadas coagulopatías y determinar si existe una correlación entre esos parámetros TEG con la presencia y cantidad de depósitos de fibrina en los capilares de diferentes órganos.

Un total de 23 caballos con trastornos frecuentemente asociados con coagulopatías (p. ej, síndrome cólico, colitis, etc.) y 5 caballos sin patologías diagnosticadas formaron parte del estudio. En estos caballos se llevó a cabo una prueba TEG menos de 24 h antes de la muerte y se realizó la necropsia antes de 24 h tras la muerte. En nuestro estudio no se encontró una correlación significativa del estado procoagulante del caballo antes de morir (determinado por TEG) y los depósitos de fibrina en secciones de riñón, hígado y pulmón (detectados mediante tinción con hematoxilina ácida fosfotúngstica —PTAH— e inmunohistoquímica —IHC—). El estudio TEG mostró que cinco caballos eran hipercoagulables (TEG_{MA} > 2SD + media) y otros cinco eran hipocoagulables (TEG_{MA} < 2SD + media). Los coeficientes de correlación (r) entre PTAH, IHC, parámetros TEG, HCT, PT, aPTT, concentración de fibrinógeno y recuento de PLT no fueron significativos. Creemos que debido al pequeño número de caballos que presentaron anomalías en la TEG y la baja tasa de depósitos de fibrina detectados, nuestro estudio careció de poder estadístico, también denominado error estadístico tipo II. Sin lugar a dudas, se necesitan más estudios para establecer si un periodo de tiempo de 24 h es adecuado para determinar si existe una correlación entre los cambios hemostáticos antes de la muerte (TEG) con los hallazgos *post mortem* (depósitos de fibrina en diferentes órganos), y si la TEG es una herramienta útil para detectar la hipercoagulabilidad en caballos y diagnosticar CID como lo es para los perros.

Por otra parte, nuestros resultados mostraron una detección mínima de depósitos de fibrina en las secciones utilizando la tinción PTAH, en comparación con los resultados obtenidos con la IHC. Esta baja correlación en cuanto a detección de depósitos de fibrina entre una tinción y otra quedó reflejada en el análisis estadístico de McNemar (x^2=506), y el coeficiente kappa de Cohen r: 0.06.

La tinción con PTAH reveló depósitos de fibrina en 4 de 82 (4,8%) de las secciones de tejido analizadas. La detección positiva se observó principalmente alrededor de los capilares de los alvéolos pulmonares. En el caso de la IHC, permitió la detección de depósitos de fibrina en 26 de 82 (31.7%) de las secciones de tejido analizadas. La detección positiva de depósitos de fibrina se observó también principalmente en

asociación a los capilares de los alvéolos pulmonares en 16 de 26 (61,5%) secciones analizadas. Curiosamente, algunas de las descripciones microscópicas de las secciones de tejido teñidas con hematoxilina y eosina identificaban depósitos de fibrina en los vasos, así como trombos de fibrina, que también fueron detectados por IHC, pero no por PTAH. Por todo ello, nuestras observaciones apuntan de nuevo a una detección más precisa de los depósitos de fibrina por IHC en comparación con la tinción de PTAH. Otro hallazgo interesante de nuestro estudio fue que 8 de los 23 caballos tenían un trastorno gastrointestinal (p. ej., síndrome cólico, salmonelosis), pero no se encontró correlación entre la detección de depósitos de fibrina y el tipo de patología. Aún más, los depósitos de fibrina detectados por IHC se encontraron con frecuencia en aquellos caballos considerados sanos por no sufrir una patología conocida. Estos resultados contradicen a los publicados en un estudio previo en el que encontraron una mayor cantidad de depósitos de fibrina en tejidos de caballos con trastornos gastrointestinales de mal pronóstico, mientras que no observaron depósitos de fibrina en caballos con cólicos de mejor pronóstico.

El análisis exhaustivo de los resultados obtenidos en los estudios expuestos anteriormente nos permite presentar las siguientes conclusiones:

1. En perros RRG la cinética de coagulación es más lenta y la fuerza del coágulo es más débil que en perros no RRG, lo que respalda la mayor tendencia a sangrar observada después de traumatismos menores o procedimientos quirúrgicos en esta raza.

2. En los perros RRG que no sangran después de la gonadectomía tiene lugar una reacción fisiológica postoperatoria con tendencia a la hipercoagulabilidad.

3. La TEG, en virtud de sus parámetros TEG_{α}, TEG_{MA} y TEG_{G}, es una prueba fiable para evaluar el riesgo perioperatorio de hemorragia en perros RRG.

4. La hipercoagulabilidad, la trombocitosis y la hiperfibrinogenemia son anomalías hemostáticas comunes en perros con carcinoma.

5. El parámetro TEG_{TG} resulta especialmente útil para diagnosticar la hiperfibrinogenemia característica del estado hipercoagulable en perros con carcinoma.

6. La TEG es una herramienta de diagnóstico útil para clasificar la etapa hipocoagulable e hiperfibrinolítica de la CID manifiesta en perros.

7. No existe correlación entre los parámetros TEG y la cantidad de depósitos de fibrina detectados por PTAH e IHC en los capilares sanguíneos de caballos. Por lo tanto, este estudio no permitió validar ningún valor pronóstico para la TEG en caballos con riesgo de coagulopatías.

8. La concordancia de las técnicas histológicas PTAH e IHC en la identificación de depósitos de fibrina en caballos es sorprendentemente pobre. De acuerdo con nuestra metodología y resultados, se recomienda el uso de IHC para la detección de depósitos de fibrina en secciones de tejidos en lugar de la PTAH.

Todos los animales incluidos en los estudios que formaron parte de esta tesis se incorporaron a los mismos tras la obtención del consentimiento firmado del propietario y la aprobación del comité ético de la correspondiente institución donde se llevaron a cabo los estudios.

SUMMARY

Hemostasis (from the Greek *heme*, blood and *stasis*, stability) is defined as stopping blood loss. However, the goal of the hemostatic system is to maintain a perfect balance between hypercoagulability, prone to thrombosis, and at the opposite end of the spectrum, hypocoagulability, prone to bleeding. In the early 1960s, a cascade-like coagulation model was proposed consisting of a series of steps in which a protease-enzyme complex activates a zymogen, that is, a coagulation factor that leads to the activation of another , which ultimately results in the generation of thrombin, which, in turn, converts fibrinogen into fibrin, a fundamental event for clot formation. The most recent reviews of the model of hemostasis emphasized the relevance of tissue factor-bearing (TF) cells, now identified as essential for thrombin formation, in a gradual coagulation process that arises after disruption of the integrity of the vascular endothelium and defined in the following steps:

a) Initiation of coagulation in TF-bearing cells.

b) Amplification of the procoagulant signal by thrombin generated in the TF carrier cell.

c) Propagation of thrombin generation on the surface of platelets.

d) Termination phase. Responsible for limiting and locating thrombin generation.

Once the vessel has been sealed, the fibrinolytic system is activated, converting fibrin into its soluble degradation products through the action of plasmin. Fibrinolysis is initiated when

plasminogen activators convert plasminogen proenzyme to plasmin by proteases associated with vascular endothelial cells. Fibrinolysis reshapes the clot and allows normal blood flow through the affected vessel.

Significant abnormalities of the hemostatic system manifest as bleeding and/or thrombosis. Bleeding can occur if there is an abnormal formation of the platelet plug and/or reduced thrombin generation during fibrin clot formation at the site of vascular injury. They are what we call primary and secondary hemostasis disorders, respectively. Bleeding can also occur if the platelet/fibrin clot breaks down prematurely due to excessive fibrinolysis, which is known as tertiary hemostasis disorder. The risk of bleeding is estimated based on the patient's medical history, physical examination, underlying disorder, and results of coagulation tests. Most common coagulation tests, including prothrombin time (PT), activated thromboplastin partial time (aPTT), and fibrinogen levels, tell us about the plasma levels of soluble coagulation factors required for hemostasis. It seems obvious that commonly used clinical clotting tests do not really reflect the complexity of hemostasis in vivo. Whole blood coagulation tests have the advantage of revealing the contributions of platelets and other circulating cells to hemostasis, and thus have the ability to assess cell/protein interactions, giving coverage to the principles stated in the theory of the cellular model of hemostasis described above.

Thromboelastography (TEG) allows an *ex vivo* analysis of the hemostatic system providing information about primary, secondary and tertiary hemostasis. In the TEG system, a cylindrical cup containing a 340 µl of whole blood sample oscillates through 4° 45′ every 5 seconds and with a pin on a torsion wire that is suspended in the blood. When the clot adheres to the pin, it transmits an electrical signal that is later represented in the form of a graph. A series of variables define this TEG graph, and these variables are in turn associated with the coagulation process. For example, TEG_R time can be related to PT and aPTT. The variables TEG_K, TEGα and TEG_{MA} resemble the fibrinogen concentration and the platelet count. Clot lysis variables LY30 and LY60 may be on par with fibrinogen breakdown products and D-dimers.

The initial development of TEG dates from the 1940s in Germany, but it was in the 1980s that TEG became a primary focus of research due to the need to overcome the limitations of common hemostatic analysis techniques for assessment of hemostasis (e.g., acquired dysfibrinogenemias, presence of coagulation inhibitors such as heparin, fibrin degradation). The results of that research led to TEG algorithms that were used to optimize the use and transfusion of blood products to coagulopathic patients.

Perioperative bleeding complications associated with specific surgical procedures (e.g., cardiac bypass, liver transplant) have been described in detail in humans; however, references in veterinary medicine are limited. Up to 26% of retired racing greyhounds (RRGs) have been shown to bleed excessively 24 to 48 hours after undergoing routine gonadectomies and/or limb amputation, to the point of requiring transfusion of blood components. Preoperative hemostasis profiles, platelet function analysis, and vWF concentration could not identify a hemostatic disorder or predict which RRG bleeds after surgery. Identifying dogs at increased risk of bleeding or simply developing a protocol for preventing excessive bleeding would be extremely valuable. To this end, we set out to comparatively analyze hemostasis in healthy RRGs and healthy non-greyhound dogs using TEG. For this, 28 RRGs and 15 non-greyhound dogs were included, all healthy and with no history of bleeding disorders or therapies that could affect hemostasis. All dogs were considered healthy based on the absence of clinical signs of disease, results of physical examination (PE), complete blood count (CBC) and hemostasis panel (aPTT, PT, fibrinogen).

Our results revealed remarkable differences between RRG and non-greyhound dogs for aPTT, hematocrit (HCT), platelet count (PLT), TEG_K, TEGα, TEG_{MA} and TEG_G. In RRG the kinetics of clot formation was slower, and the clot strength was weaker than in non-greyhound dogs. These results were in accordance with the tendency to bleed observed after minor trauma or surgical procedures in RRG. The high HCT and low PLT count are nothing more than well-known hematological features of RRGs, which speaks to their unique rheological properties. On the other hand, the high viscosity of whole blood in RRG (HCT in non-greyhound dogs: 41.81% vs. 47.61% in

RRG) could cause a decrease in TEG_{MA} and TEG_G parameters due to the restriction of movement of the transducer pin in the cylindrical cup of the TEG. Also, since RRGs have less plasma per unit volume, lower TEG_{MA} and TEG_G values may be the result of excessive anticoagulant addition. However, according to the Guidelines of the US National Committee for Clinical Laboratory Standards for the collection, transport, and processing of blood samples to analyze the plasma-based coagulation assay, the anticoagulant concentration should only be adjusted in patients with HCT> 55%. Only 2 RRGs in this study had HCT> 55% (55.7 and 59%).

Once the hemostatic peculiarities of RRG were characterized through the TEG, we were able to tackle the next step by identifying the perioperative hemostatic singularities in RRG. A total of 21 healthy RRG dogs with no history of bleeding disorders or abnormalities in PE, CBC, serum biochemistry profiles (in dogs older than 5 years), and negative SNAP test (SNAP-4DX; IDEXX Laboratories) for common vector-borne diseases were included in the study. When studying perioperative bleeding disorders in RRG undergoing gonadectomies, we found no significant differences between "bleeding" and "non-bleeding" for the preoperative parameters of TEG, aPTT, fibrinogen concentration, PLT count, or hemoglobin concentration (HGB). However, the increase in TEG_{MA} and TEG_G parameters observed in non-bleeding RRGs suggests the formation of a stronger clot in this group, which is interpreted as a supportive response to the physiological hypercoagulability induced by inflammation or stress associated with surgery. To the best of the authors' knowledge, this study was a pioneer in documenting a reactive phase of postoperative hypercoagulability in dogs. It is also possible that since RRG bleeding syndrome is due to a faulty interaction of cells and proteins, conventional quantitative coagulation tests (e.g., PLT count) and those based on blood plasma (e.g., aPTT, PT, fibrinogen concentration) are not adequate to diagnose the RRG bleeding syndrome. In our study, TEG was the only diagnostic test (for TEGα, TEG_{MA}, and TEG_G parameters) that was positively correlated with clinical signs of postoperative bleeding. Therefore, TEG may be a useful test to assess the perioperative risk of bleeding in RRG.

Thereafter, we focused our research on the correlation between cancer and coagulopathies. Hypercoagulability in cancer has been related to the tumor's ability to over-express procoagulants, such as TF, through the interaction of tumor cells with platelets, macrophages-monocytes, and vascular endothelial cells. This phenomenon is called the pro-thrombotic cell phenotype. Several human studies have shown a strong association between idiopathic venous thrombosis and subsequent development of clinical cancer and the prevalence of thrombosis, up to 30%, in cancer patients. It is obvious that identifying dogs in a prothrombotic state to establish treatments that reduce the risk of thrombosis may be of clinical interest in veterinary medicine. Thus, we established the following objective to analyze the hemostatic characteristics in dogs with carcinomas using TEG and other markers of coagulopathies such as PAI-1 (Plasminogen Activator Inhibitor) and TAT (Thrombin-Antithrombin complexes).

In this study we included a total of 32 dogs with carcinomas with no history of having received anticancer therapy and/or COX (cyclooxygenase) inhibitors at least 2 weeks before sampling. Dogs with carcinomas showed faster generation of thrombi (TEG_G 834.8 ± 91.1 vs. 707.8 29 ± 75.8 mm / min; mean ± SE), increased fibrinogen concentration (276 vs. 151 mg / dL) and PLT count (425 vs. 324 x 109 U / L), but lower PAI-1 activity (15.7 vs. 26.2 IU / ml). The most common hemostatic abnormalities found in dogs with carcinoma were: hypercoagulability (TEG_G> mean + 2SD of healthy dogs) in 15/32 (46%), thrombocytosis (PLT> 424 x 109 U / L) in 15/32 (46%), and hyperfibrinogenemia (fibrinogen> 384 mg / dl) in 9/32 (28%). Hyperfibrinogenemia appears to be present in the hypercoagulable state in dogs with carcinoma, since 61% of hypercoagulable dogs with carcinoma were hyperfibrinogenic. As expected, clotting times (PT, aPTT) were similar between dogs with and without carcinoma, supporting the lack of ability of plasma hemostatic assays to detect hypercoagulability.

TAT complexes are created after thrombin production and have been proposed as a subclinical marker of thrombin formation. Our results, however, did not demonstrate differences between dogs with

carcinoma and without carcinoma for the formation of TAT complexes. PAI-1 and other components of the fibrinolytic system play an important role as modulators of angiogenesis and tumor metastases, but there is still controversy about the biological relevance of this protein in tumorigenesis. For example, a high expression of PAI-1 is associated with a poor prognosis in various types of cancer in humans. However, in mice the lower concentrations of PAI-1 increased angiogenesis up to three times, but at high concentrations they inhibited it. In our study, the PAI-1 activity in dogs with carcinoma was significantly less than in the control group, a striking result that encourages further research on the role that this molecule plays in tumor progression.

onlooking into another aspect of coagulopathies and cancer, the next step was the study of DIC (Disseminated Intravascular Coagulation) using TEG. DIC can be described and categorized in two stages: the initial "non-overt" stage is dominated by hypercoagulability; due to the continuous consumption of clotting factors and platelets that takes place during the initial stage, a level is reached where the integrity of normal hemostatic function cannot be maintained, leading to a state of hypocoagulability or "overt" stage, where clinical signs of spontaneous bleeding can be observed in the patient. For this study, we used a 10-year-old Bichon Frise dog in which a diagnosis of visceral hemangiosarcoma with lung metastasis had been established. The analysis of hemostasis revealed a prolonged clotting time (i.e., very high PT and aPTT outside the range), hypofibrinogenemia, and an increase in D-dimers, so a presumptive diagnosis of DIC was made based on these findings. TEG plots were positively correlated with the results of previous coagulation tests showing hypocoagulability (i.e., prolonged TEG_K - clot kinetics; weaker clot with a low TEG_{MA}) and hyperfibrinolysis (i.e., increased TEG_{LY30} and TEG_{LY60}). Based on these results, we determined that the dog was in the hyperfibrinolytic phase of DIC and, therefore, that TEG is a valuable diagnostic test for diagnosis in dogs. The fibrinolytic parameters provided by TEG (i.e., TEG_{LY30} and TEG_{LY60}) can add important information for the diagnosis of DIC, but also for its treatment and prognosis.

Our latest research study focused on equines suffering from common diseases frequently associated with coagulopathies. Accumulative fibrin deposits in organ capillaries (e.g., kidney, liver, lung) have been suggested to be linked to the hypercoagulable state observed in DIC. TEG could be extremely useful to identify horses in risk of developing DIC in advance or at least before large deposits of fibrin occur in the capillaries of the organs, leading to multiple organ failure. Early diagnosis could allow early interventions with blood products, the establishment of an accurate prognosis. Therefore, our objective was to investigate the hemostatic characteristics in horses with diseases that usually have associated coagulopathies using TEG, and to determine if there is a correlation between these TEG parameters with the presence and amount of fibrin deposits in the capillaries of different organs.

A total of 23 horses with disorders frequently associated with coagulopathies (e.g., colic syndrome, colitis) and 5 horses without diagnosed pathologies were part of the study. A TEG test was done less than 24 h before death, and necropsy was done no later than 24 h after death. In our study, no significant correlation was found between the procoagulant state of the horses before death (determined by TEG) and fibrin deposits in sections of the kidney, liver and lung (detected by staining with phosphotungstic acid hematoxylin -PTAH- and immunohistochemistry -IHC-). The TEG study showed that five horses were hypercoagulable (TEG_{MA} > 2SD + mean) and another five were hypocoagulable (TEG_{MA} < 2SD + mean). Correlation coefficients (r) between PTAH, IHC, TEG, HCT, PT, aPTT parameters, fibrinogen concentration, and PLT count were not significant. We believe that due to the small number of horses that presented abnormalities in the TEG and the low rate of fibrin deposits detected, our study lacked statistical power, also called type II statistical error. Undoubtedly, more studies are needed to establish whether a 24-hour time period is adequate to determine whether there is a correlation between hemostatic changes before death (TEG) with post-mortem findings (fibrin deposits in different organs), and whether TEG is a useful tool for detecting hypercoagulability in horses and diagnosing DIC as it is for dogs.

Furthermore, our results showed minimal detection of fibrin deposits in the sections using PTAH staining, compared to the results obtained with the IHC. This low correlation in terms of detection of fibrin deposits between one stain and another was reflected in the McNemar exact test (x^2=506; $p<0.01$), and the Cohen's kappa coefficient was 0.06. PTAH staining revealed fibrin deposits in 4 of 82 (4.8%) of the tissue sections analyzed. Positive detection was observed mainly around the capillaries of the pulmonary alveoli. In the case of the IHC, it allowed the detection of fibrin deposits in 26 of 82 (31.7%) of the analyzed tissue sections. Positive detection of fibrin deposits was also observed mainly in association with the capillaries of the pulmonary alveoli in 16 of 26 (61.5%) sections analyzed. Interestingly, some of the microscopic descriptions of the hematoxylin and eosin stained tissue sections identified fibrin deposits in the vessels, as well as fibrin thrombi, which were also detected by IHC, but not by PTAH. For all these reasons, our observations point to a more precise detection of fibrin deposits by IHC compared to PTAH staining. Another interesting finding from our study was that 8 of the 23 horses had a gastrointestinal disorder (e.g., colic syndrome, salmonellosis), but no correlation was found between the detection of fibrin deposits and the type of pathology. Furthermore, fibrin deposits detected by IHC were frequently found in those horses considered healthy because they did not suffer from a known pathology. These results contradict those published in a previous study in which they found a greater amount of fibrin deposits in tissues of horses with gastrointestinal disorders with a poor prognosis, while fibrin deposits were not observed in horses with colic of better prognosis.

The following conclusions were stated based on the results obtained from the multiple studies presented above allows us to present the following conclusions:

1. In RRG dogs, clotting kinetics are slower and clot strength is weaker than in non-RRG dogs, supporting the increased bleeding tendency observed after minor trauma or surgical procedures in this breed.

2. In RRG dogs that do not bleed after gonadectomy, a postoperative physiological reaction with a tendency to hypercoagulability occurs.

3. TEG, by virtue of its TEGα, TEG_{MA} and TEG_{G} parameters, is a reliable test to assess the perioperative risk of bleeding in RRG dogs.

4. Hypercoagulability, thrombocytosis, and hyperfibrinogenemia are common hemostatic abnormalities in dogs diagnosed with carcinoma.

5. The TEG_{TG} parameter-parameter is especially useful for diagnosing hyperfibrinogenemia characteristic of the hypercoagulable state in dogs with carcinoma.

6. TEG is a useful diagnostic tool to classify the hypocoagulable and hyperfibrinolytic stage of "overt" DIC in dogs.

7. There is no correlation between TEG parameters, and the amount of fibrin deposits detected by PTAH and IHC in capillaries of horses. Therefore, this study did not validate any prognostic value for TEG in horses at risk of coagulopathies.

8. The agreement between PTAH and IHC histological techniques in the identification of fibrin deposits in horses is surprisingly poor. In accordance with our methodology and results, the use of IHC is recommended for the detection of fibrin deposits in tissue sections instead of PTAH.

All the animals were included in the studies after obtaining the signed consent of the owners and the approval of the ethical committee of the corresponding institution where the studies were carried out.

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LIST OF ABBREVIATIONS

α	*Angle: angle of the TEC trace*
ADP	*Adenosine Diphosphate*
APC	*Activated Protein C*
aPTT	*Activated Partial Thromboplastin Time*
AT	*Antithrombin*
ATP	*Adenosine Triphosphate*
CBC	*Complete Blood Count*
CV-CVi	*Coefficient of Variation*
COX	*Cyclooxigenase*
DIC	*Disseminated Intravascular Coagulation*
EPM	*Equine Protozoal Myeloencephalitis*
F	*Factor*
FDP	*Fibrin Degradation Products*
Fib/FIB	*Fibrinogen*
G	*G value: shear modulus strength*
GP	*Glycoprotein*
HCT	*Hematocrit*
H&E	*Hematoxylin and Eosin*
HGB	*Hemoglobin*
HSA	*Hemangiosarcoma*
IHC	*Immunohistochemistry*
IL	*Interleukin*
ITP	*Immune Mediated Thrombocytopenia*

K	*K-time: clot kinetics*
LY30 / LY60	*Lysis percentage at 30-60 minutes after reaching MA*
MA	*Maximum Amplitude*
OHE	*Ovariohysterectomy*
OSU	*Ohio State University*
PA	*Plasminogen Activator*
PAF	*Platelet Activating Factor*
PAI	*Plasminogen Activator Inhibitor*
PAR	*Plasminogen Activator Receptor*
PBS	*Phosphate Buffered Saline*
PE	*Physical Examination*
PFA	*Platelet Function Analyzer*
Plt / PLT	*Platelet*
PT	*Prothrombin Time*
PTAH	*Phosphotungstic Acid Hematoxylin*
PVS	*Paulo Vilar Saavedra*
r-TEG	*Rapid TEG*
R	*R-time: reaction time*
RCF	*Ristocetin Co-Factor*
ROTEM	*Rotational Thromboelastometry*
RRG	*Retired Racing Greyhounds*
SD	*Standard Deviation*
TAFI	*Thrombin Activatable Fibrinolysis Inhibitor*
TAT	*Thrombin Anti-Thrombin complexes*
TEG	*Thromboelastograph – Thromboelastography*
TEG–PM/ PM	*Thromboelastography Platelet Mapping*

TF	*Tissue factor*
TFPI	*Tissue Factor Pathway Inhibitor*
TxA2	*Thromboxane A2*
VE	*Vascular Endothelium*
VEGF	*Vasculo-Endothelial Growth Factor*
vWF	*von Willebrand Factor*

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1. INTRODUCTION & LITERATURE REVIEW

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1. INTRODUCTION & LITERATURE REVIEW

Hemostasis (from the Greek *haemo*, blood, and *stasis*, stoppage) is defined as the arrest of blood loss.[1] However, the final goal of the hemostatic system is to maintain a perfect equilibrium between hypercoagulability, with a tendency towards thrombosis, and the opposite end of the spectrum or hypocoagulability, with a tendency towards bleeding.[2] A basic understanding of hemostasis and the physiology of the hemostatic system is important to interpret correctly the diagnostic tests used to evaluate coagulation and to provide the correct diagnosis or treatment plan for a patient with a suspected hemostatic abnormality.

1.1 COMPONENTS AND FUNCTION OF THE HEMOSTATIC SYSTEM

1.1.1 Vascular endothelium

The largest of the organs involved in hemostasis, the vascular endothelium (VE) consists of a monolayer of cells that builds up a tapetum conferring separation of circulating blood from sub-endothelial structures of the vessel wall. Some of the multiple functions assigned to VE are: active regulator of blood pressure and vasomotor tone, angiogenesis, cell adhesion-transmigration, inflammatory and immune responses, permeability and, most importantly in this review, hemostasis by expression of thromboregulatory molecules.[3-5] The phenotypical answer observed in endothelial cells that allows for most of the VE functions described above is mediated by translational —rapid control of response— rather than transcriptional control pathways.[6]

1.1.2 Platelets

Also called thrombocytes, platelets are non-nuclear cellular elements derived from fragmentation of megakaryocytes. Platelets are released into circulation from the bone marrow at the end stage of thrombopoiesis and have an average life span of 5-8 days. Platelets measure 1.5-3.0 μm in diameter and count for 150-300x10^3 U/μL of blood.[7] A representation of the structural components of the platelets is shown in Figure1. Following an external to internal succession of morphological structures, platelets are composed by:

a) Glycocalix: a negative net charged coat of membrane glycoproteins, glycolipids, mucopolysaccharides, and adsorbed plasma proteins.[8]

b) Plasma membrane: an enriched with arachidonic acid phospholipidic bilayer unit with integrated cholesterol, glycolipids and glycoproteins.[8] The negatively charged phospholipids are mostly present in the inner leaflet, then, separated from the plasma coagulation factors to prevent inappropriate activation coagulation.[8,9]

c) Cytoskeleton: constituted by three major structures that shape up the discoid aspect of the platelet: spectrin membrane skeleton, microtubule coil and the actin cytoskeleton.[10]

d) Organelles:

I) Peroxisomes: contribute to lipid metabolism, especially to plasmalogen synthesis, and may participate in the synthesis of platelet-activating factor (PAF).[11]

II) Mitochondria: involved in oxidative energy metabolism and life span through the BCL-2 apoptotic family proteins (i. e., BCL-XL and BAK).[12,13] Adenosine Tri-Phosphate (ATP) consumption by platelets is mainly committed to maintain homeostasis (i.e., ionic and osmotic) and continuous polymerization and depolymerization of actin.[14] Depleting platelets of their metabolic pool of ATP and Adenosine Di-Phosphate (ADP) decreases their responses to stimuli, but the effects are not uniform: shape change is only

minimally affected, whereas there is an increasingly significant effect on aggregation, alpha and dense granules secretion, arachidonic acid liberation, and lysosome secretion.[15,16]

III) Dense granules: designated as the metabolic and storage pools of adenine nucleotides.[13] There is more ADP than ATP in the dense granules (ATP to ADP ratio is 2:3), which is the reverse of their relative concentrations in the cytoplasm (ATP to ADP ratio is 8:1). The intrinsic electron density of dense granules when viewed as unstained whole mounts derives from their high content of calcium and serotonin.[13,17]

IV) Alpha granules: defined by the morphologic features observed by electron microscopy, it is the most abundant of the secretory granules and critical for platelet function. Alpha granule function derives from its heterogenous content: Factor V, fibrinogen, integrins, glycoprotein receptors, P-selectin, Vasculo-Endothelial Growth Factor (VEGF) and von Willebrand Factor (vWF) are some of the described contents. As in general, alpha granules concentrate large polypeptides that contribute to primary and secondary hemostasis.[18-20]

e) Other substances:

I) Platelets contain high concentrations of VEGF, an important stimulator of angiogenesis, released under wound healing conditions and found in elevated concentrations in malignancies. Therefore, a cancer biomarker too.[21-23]

II) Factor XIII is present in the cytoplasm of platelets accounting for 50% of total blood Factor XIII.[24] It differs from plasma Factor XIII in having only the alpha subunits.[24,25] Upon platelet activation, Factor XIII redistributes to the platelet periphery where it associates with the cytoskeleton and crosslinks filamin and vinculin in a transglutaminase reaction.[26]

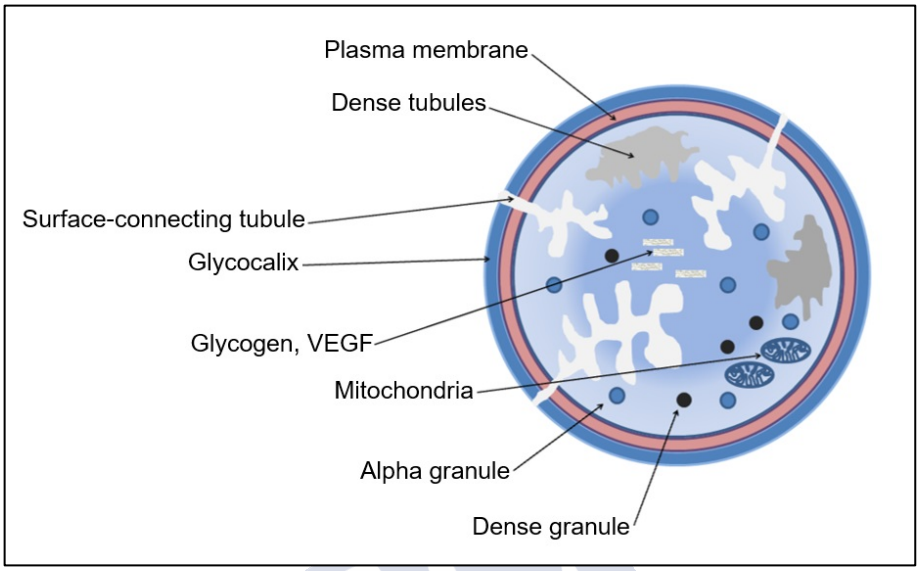

FIGURE 1. Representation of the structural components of a platelet. Source: modified from G. Beards, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=21906052

Platelet composition and function is determined by factors endocytosed from the circulation. Platelets serve as a depot of multiple molecules that affect platelet function, cell proliferation, fibrinolysis, inflammation, innate immunity, vascular tone, and wound healing; these agents are actively released upon platelet activation. Platelet's main function is to provide the initial seal to stop bleeding from a vessel injury. In a stepwise, collagen fibrils within the vessel wall become exposed to the circulation when the endothelial cell monolayer is disrupted. The exposed matrix presents specific receptors that form a complex with vWF. Platelets tumbling on the periphery of the rapidly moving bloodstream are captured when glycoprotein (GP) VI and αIIβ1 receptors on the platelet surface bind to the collagen component of the extracellular matrix. In addition, GP Ib on the platelet surface binds to the vWF A1 domain, establishing contacts that slow the forward progress of the platelet long enough for platelet activation to occur. Drivers for platelet activation include the signaling events that occur downstream of receptors for collagen (GP VI and GP Ib), thrombin (PAR1 and PAR4), adenosine diphosphate (P2Y1 and P2Y12), and

thromboxane A2 (TxA2-TP). Following this initial tethering of the platelet to the vessel wall, subsequent firm adhesion results in signal transduction within the platelet and flattening of the initially round or "plate" looking platelets. Secondary to firm adhesion, the activated platelets bound within the thrombus will begin to incorporate new platelets from circulation through platelet-platelet interactions mediated by the integrin receptor αIIbβ3. Additionally, circulating platelets and loosely associated platelets will become activated through positive feedback initiated through the formation of secondary signals via the oxygenases COX-1 and 12-LOX as well as through granule secretion of small molecules known to activate the platelet. The resulting platelet thrombus will therefore consist of a "core" of tightly packed P-selectin positive platelets surrounded by a "shell" of loosely packed platelets that require secondary feedback through various receptors.[7,27-29] Sequential steps of hemostasis are shown in Figures 2 & 3.

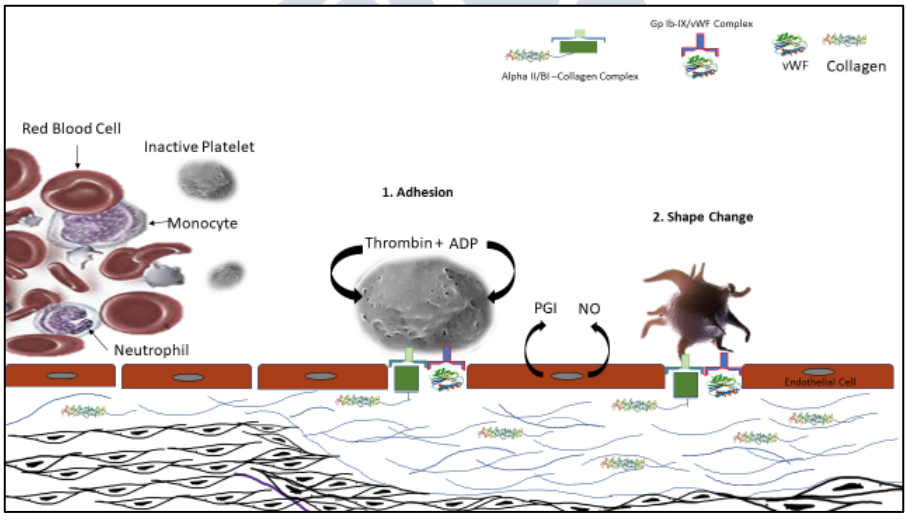

FIGURE 2. Sequential steps (left to right) of primary hemostasis consisting of initial platelet activation, vasoconstriction, adhesion and subsequent change in shape of the activated platelet. ADP: Adenosine Di-Phosphate; NO: Nitrogen Oxide; PGI: Prostaglandin; TF: Tissue Factor. Source: own elaboration.

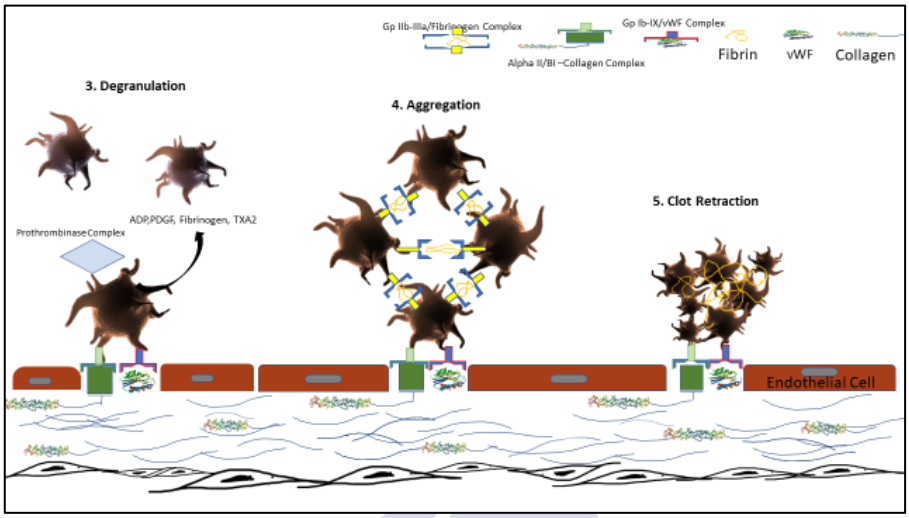

FIGURE 3. Sequential steps (left to right) of secondary hemostasis consisting of thrombin production, fibrin generation, platelet aggregation, stabilization and clot retraction. ADP: Adenosine Di-Phosphate; PDGF: Platelet Derived Growth Factor; TXA2: Thromboxane A2. Source: own elaboration.

1.1.3 Coagulation system: clotting factors and cell-based model of coagulation

In its simplest definition, coagulation rends to generate thrombin that converts soluble fibrinogen to fibrin. In the early 60s it was proposed a cascade-like model of coagulation to occur as a sequential series of steps in which a protease-enzyme complex activates a zymogen, this is, a clotting factor leading to the activation of another, finally resulting in a burst of thrombin generation. The enzyme complexes are composed of vitamin K-dependent enzymes, members of the serine protease family, and a non-enzyme cofactor assembled on anionic phospholipid membranes in a calcium-dependent fashion. Each activated substrate (enzyme complex) becomes the enzyme component of the subsequent complex, which means that strong physiological reactions originate from a small boost.[30] There are two categories of procoagulant cofactor proteins: the cell-bound cofactors (Tissue Factor and thrombomodulin) and the soluble plasma-derived procoagulant

procofactors (Factor V and Factor VIII with its circulating carrier vWF).[31]

I) Cell-bound cofactors: Tissue Factor (TF) is a transmembrane protein that functions as a non-enzymatic cofactor for Factor VIIa in the extrinsic tenase complex.[32] Thrombomodulin is a type 1 transmembrane protein constitutively expressed on the surface of vascular endothelial cells. Thrombomodulin is a high-affinity receptor for all thrombin forms and acts as a cofactor for the thrombin-dependent activation of Protein C.[33,34]

II) Soluble plasma procofactors: Factor V is a large single-chain GP found in plasma (75-82%) and in the alpha granules of platelets (18-25%).[35] The procofactor Factor V is proteolyzed by α-thrombin to the active cofactor Factor Va.[36] Factor Va functions both as a Factor Xa receptor and positive modulator of Factor Xa catalytic potential in the prothrombinase complex.[37] Factor Va is proteolytically inactivated by Activated Protein C (APC).[38]

The soluble procofactor Factor VIII circulates in plasma in complex with the large multimeric protein vWF.[39] vWF acts to regulate the plasma concentration of Factor VIII. After forming, vWF–free Factor VIIIa forms a complex with the serine protease Factor IXa, Ca^{2+}, and a membrane (provided by platelets) resulting in the intrinsic tenase complex. vWF has several key roles in coagulation. It is synthesized in endothelial cells and also contained in the alpha granules of platelets.[40] vWF is a large adhesive GP that has binding sites for Factor VIII, heparin, collagen, platelet GP Ib, and platelet GP IIb–IIIa.[41,42] vWF acts as the bridge between platelets to promote platelet aggregation. The primary platelet binding site for vWF is the GP Ib–IX–V receptor complex. GP Ib–IX–V is an active receptor on unstimulated platelets and serves to promote platelet aggregation and adhesion to vWF in the absence of platelet activation.[43] A graphic representation of the tenase and phrothombinase complexes, and interaction of factors, cells and platelets to elaborate thrombin is shown in Figure 4.

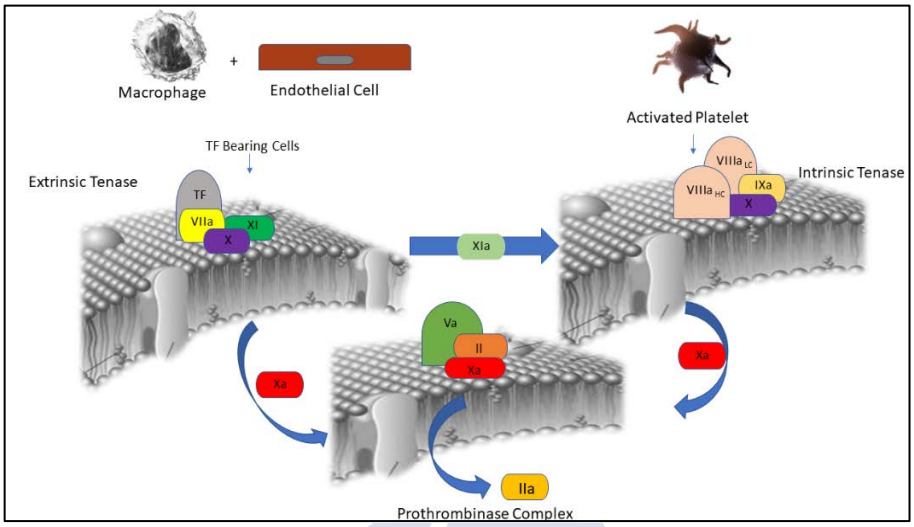

FIGURE 4. Graphic representation of the Extrinsic Tenase (TF: Tissue Factor, VIIIa, XI, X), Intrinsic Tenase ($VIIIa_{LC}$, $VIIIa_{HC}$, IXa, X) and Phrothrombinase Complex (Va, II, Xa). TF-bearing cells (Macrophage & Endothelial Cell) and activated platelets provide a substrate-membrane for the complexes and coagulation factors resulting in elaboration of Factor IIa (Thrombin). Source: own elaboration, inspired by Figure 122.4 in Hematology: Basic Principles and Practice 7th Ed. Chapter 122: Overview of Hemostasis and Thrombosis by Fredenburgh, J.C and Weltz, J.I. by Elsiever, Inc.[44]

The coagulation cascade has been explained using a theoretical Y-shaped scheme, based on two separated pathways (extrinsic and intrinsic) that converge into a common-pathway. Hoffman and Monroe[44] proposed a cell-based model of physiology that emphasizes the relevance of TF-bearing cells in a stepwise coagulation process that occurs as outlined in the following sections and as shown in Figures 4 & 5:

a) Initiation of coagulation on TF-bearing cells. Responsible for generating the initial burst of thrombin required for platelet and coagulation factors activation.

b) Amplification of the procoagulant signal by thrombin generated on the TF-bearing cell. Responsible for the explosive

thrombin generation necessary to sustain the coagulant response.

c) Propagation of thrombin generation on the platelet surface.

d) Termination phase. Responsible for limiting and localizing thrombin generation.[45]

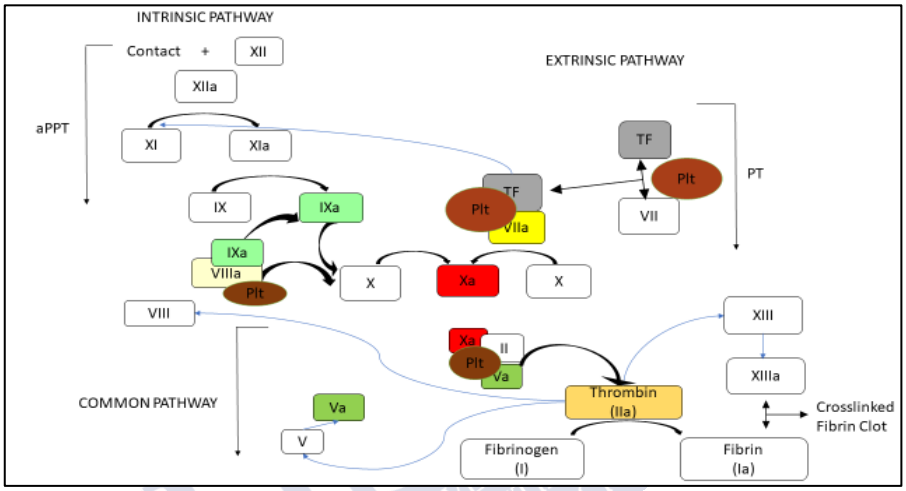

FIGURE 5. Two separated pathways (extrinsic and intrinsic) converge into a common pathway. aPTT (activated Partial Thromboplastin Time); PT (Prothrombin Time); TF: Tissue Factor; Plt: Platelet. Source: modified from J. D., CC BY-SA 3.0, https://en.wikipedia.org/wiki/Coagulation

A rupture of the integrity of the vascular endothelium constitutes the initiation of a localized prothrombotic state. The small amount of thrombin generated at the rupture site activates non-enzyme cofactors and platelets. Then, activated platelets provide a negative charged surface on which the enzyme complexes gather to stimulate rapid thrombin generation. Following Monroe's model of hemostasis, the extrinsic pathway consists of Factor VIIa/TF complex working with Factor Xa/Va complex, and the intrinsic pathway as Factor XIa working with the complexes of Factors VIIIa/IXa and Factors Xa/Va. The extrinsic pathway operates on the TF-bearing cell to initiate and amplify coagulation. In consequence, TF exposure on monocytes, endothelial and extravascular cells are recognized key steps. By contrast, the intrinsic pathway operates mainly on the activated platelet surface to

produce the burst of thrombin that causes formation and stabilization of the fibrin clot of coagulation (propagation), and where the extrinsic and intrinsic pathways are not redundant.

1.1.3.1 Inhibitory regulation of the coagulation system

Coagulation is built up over an all-or-none response system with a threshold that responds to levels of stimuli. The regulation of those stimuli is starred by cellular and humoral anti-coagulant mechanisms to prevent thrombin generation (locally and systemically). Antithrombin (AT) inhibits thrombin and Factors XIIa, XIa, Xa and IXa. Heparin and analogs empower AT effects. Tissue Factor Pathway Inhibitor (TFPI) neutralizes the extrinsic coagulation pathway Factors Xa and VIIa. In addition, APC induces proteolytic inactivation of Factors VIIIa and Va, as well as direct cell-signaling activities involving protease activated receptors 1 and 3, endothelial cell Protein C receptor, integrin CD11b/CD18, and apolipoprotein E receptor 2. Plasma protease inhibitors also neutralize various coagulation proteases.[46]

1.1.4 Fibrinolytic system

Once the vessel has healed, the fibrinolytic system is activated, converting fibrin to its soluble degradation products through the action of plasmin.[47] Fibrinolysis initiates when plasminogen activators convert proenzyme plasminogen to plasmin by proteases associated with the vascular endothelial cells. There are two plasminogen activators (i.e., t-PA and u-PA) in blood. t-PA has little enzymatic activity *per se*, but its activity increases by at least three orders of magnitude when fibrin is present, which means fibrin serves as a template that binds t-PA and plasminogen and promotes their interaction. Vascular endothelium is the principal source of t-PA *in vivo* and it is localized primarily in small blood vessel. Therefore, t-PA mediates intravascular fibrin degradation. Instead, u-PA binds to a specific u-PA receptor (u-PAR) on the surface of cells, where it activates cell-bound plasminogen. Consequently, pericellular proteolysis during cell migration and tissue remodeling and repair are the major functions of u-PA.[48]

Fibrinolysis is subjected to precise control because of the actions of multiple activators, inhibitors, and cofactors.[49] Beyond its more traditional role in fibrin degradation, the fibrinolytic system also supports a variety of tissue remodeling mechanisms. The solubilization and removal of the fibrin scaffolding of the hemostatic plug is coordinated with the processes of tissue repair and regeneration in part triggered by products of the processes. The extracellular matrix is degraded to allow for cell migration into the damaged area. Vascular cells repopulate the site and recreate the elements necessary to restore the vessel to its previously unperturbed state.[5]

1.1.4.1 Inhibitory regulation of the fibrinolytic system

Because of the multiple roles of fibrinolysis and therefore sites of action, regulation of fibrinolysis occurs at multiple levels. The serpins, Plasminogen Activator Inhibitor - 1 (PAI-1), and, to a lesser extent, Plasminogen Activator Inhibitor - 2 (PAI-2), inhibit the plasminogen activators, whereas α2-antiplasmin deactivates circulating plasmin blocking the active site.[48] Because plasmin binds to fibrin via its kringle domains, plasmin generated on the fibrin surface resists inhibition by α2-antiplasmin. Endothelial cells synthesize PAI-1, which inhibits both t-PA and u-PA, whereas monocytes and the placenta synthesize PAI-2, which specifically inhibits u-PA.[49,50]

Thrombin-activatable fibrinolysis inhibitor (TAFI) also modulates fibrinolysis. TAFIa attenuates fibrinolysis by removing fibrin-binding sites for plasminogen, plasmin, and t-PA, attenuating activation, and promoting inhibition. TAFI links fibrinolysis to coagulation because the thrombin–thrombomodulin complex not only activates TAFI, which attenuates fibrinolysis, but also activates Protein C, which silences thrombin generation.[47]

Expression of u-PA mRNA in vascular endothelium is strongly stimulated during wound repair and physiologic angiogenesis (e.g., within ovarian follicles, corpus luteum).[5,51] The association of u-PA with the blood vessel wall appears to reflect its association with the u-PAR and a variety of nonproteolytic functions such as directed cell migration, cellular adhesion, differentiation, and proliferation.[5]

1.2 ANALYTIC APPROACH TO COMMON HEMOSTATIC ABNORMALITIES

The risk for bleeding assessment is based on the bleeding history, physical examination, suspected underlying disorder, type and site of surgical procedure, and results of hemostatic test (e.g., Prothrombin Time - PT, activated Partial Thromboplastin Time - aPTT, platelet count - PLT count).

1.2.1 Analysis of thrombocytopenias and platelet function disorders

It is of clinical relevance to address an abnormal low platelet count report and differentiate between pseudothrombocytopenia (e.g., platelet clumping) *vs.* real thrombocytopenia induced by decreased platelet production (e.g., bone marrow suppression, myelophthisis, hereditary disorders, macrothrombocytopenia MYH9, vitamin B12 or folic acid deficiency), platelet consumption (e.g., Disseminated Intravascular Coagulation - DIC) and/or platelet destruction (e.g., Immune mediated Thrombocytopenia - ITP, thrombotic thrombocytopenic purpura).[52,53] When analyzing platelet dysfunction, some of the most commonly used test include: Platelet Function Analyzer (PFA), Ristocetin Co-factor Activity assay, platelet aggregation, and clot retraction.[53,54]

1.2.2 Analysis of coagulation factors deficiencies

PT-based assays serve to measure the activities of Factors II, V, VII, and X. aPTT-based assays serve to measure activities of Factors VIII, IX, XI, and XII, prekallikrein, and high-molecular-weight kininogen. The plasma level of fibrinogen is most commonly measured indirectly by assessing the time required for clot formation in the patient's diluted plasma (Clauss method). [55] The Ristocetin Co-factor activity (i.e., RCF function) of vWF can be measured by the ability of the patient's plasma to support the agglutination of a suspension of formaldehyde-fixed normal platelets by ristocetin.[54]

The most common assays analyze the ability of dilutions of the patient's plasma to correct the clotting time of plasma known to be deficient in the factor being measured (substrate plasma). The results

are compared to the ability of dilutions of normal reference plasma to correct the abnormality in the substrate plasma. From there, to depict specific coagulation factor deficiency with immunologic assays (enzyme-linked immunosorbent assay using specific polyclonal or monoclonal antibodies to assess the presence of the factor, independent of its function.[56,57]

Crossed immunoelectrophoresis measures both the immunologic reactivity and the mobility of the protein in an electric field; thus, it can detect protein abnormalities that affect electrophoretic migration. [58] The abnormalities include the presence of antibody–antigen complexes that migrate differently from the protein itself, such as antiprothrombin–prothrombin complexes in patients with systemic lupus erythematosus or antiphospholipid syndrome.[59]

The presence of inhibitors is suspected by the occurrence of prolonged PT or aPTT performed on a 1:1 mixture of the patient's plasma and normal plasma, but further studies help define the nature of the inhibitor and its titer (heparin in the sample).[57,60]

1.3 WHOLE BLOOD VISCOELASTIC COAGULATION TEST: THROMBOELASTOGRAPHY AND ROTATIONAL THROMBOELASTOMETRY

All common coagulation tests including PT, aPTT, thrombin clotting time, fibrinogen levels, and coagulation factor levels tell us something about the plasma level —quantity— of soluble factors required for hemostasis. However, bleeding can occur if there is abnormal platelet plug formation and/or reduced thrombin generation and subsequent fibrin clot formation at the site of vascular injury; this is, disorders of primary and secondary hemostasis, respectively. Bleeding also can occur if the platelet/fibrin clot is untimely degraded because of excessive fibrinolysis.

It seems obvious that commonly used clinical coagulation tests do not really reflect the complexity of hemostasis *in vivo*. Several studies indicate that unselected coagulation tests have no significant predictive

value of perioperative bleeding.[61,62] Whole blood coagulation tests have the advantage that they may reflect the contributions of platelets and circulating cells to the hemostatic process and, therefore, the ability to evaluate cell/protein interaction and to cover most of the principles enounced in the cell-based theory of hemostasis.[45]

Thromboelastography (TEG) allows for an *ex vivo* analysis of the hemostatic system providing information about primary hemostasis, secondary hemostasis and the fibrinolytic system. In the TEG system, a cylindrical cup containing a 340 µl whole blood sample oscillates through 4° 45′ every 5 seconds and a pin on a torsion wire is suspended in the blood. As the viscoelastic strength of the clot increases, more rotation is transmitted to the torsion wire and is detected by an electromagnetic transducer.[63] A series of variables define the TEG tracing and describe aspects of the coagulation process (Figure 6). For example, R-time (R) can be compared with PT and aPTT. K-time (K), angle (α) and Maximal Amplitude (MA) variables analogize with fibrinogen concentration and PLT count. LY30 and LY60 clot lysis variables can be on a par with Fibrinogen Degradation Products (FDP) and D-dimers.[63,64] Since TEG employs methodology that is extremely different, then the question is raised as to what extent the TEG parameters can reflect the changes of corresponding conventional tests.[64]

TEG was developed in the Germany of the 1940s, but first time described for detection of coagulopathies and abnormal fibrinolysis in patients undergoing liver transplantation by researchers of the University of Pittsburgh in the 1980s.[65] The 80's investigation in the use of TEG responded to the need for overcoming limitations of hemostasis evaluation (e.g., acquired dysfrinogenemias, presence of coagulation inhibitors like heparin, fibrin degradation) when using common hemostatic test (e.g., fibrinogen levels, aPTT, PT, D-dimers).[65] The results of that investigation were TEG algorithms that currently guide transfusion of blood products with consequent less usage of blood derivates.[66] The use of those algorithms was extended to other invasive procedures that involved coagulopathy, most notably

cardiovascular surgery, with similar advantage, and at present it is used for a more accurate assessment of the coagulation status of the patient undergoing cardiac bypass surgery.[67,68-71] Nowadays, we accept that TEG allows rapid identification of some defects in clotting factor concentration, platelet function, and thrombin generation and/or fibrinogen concentration that are not always detectable by routine coagulation testing.[65]

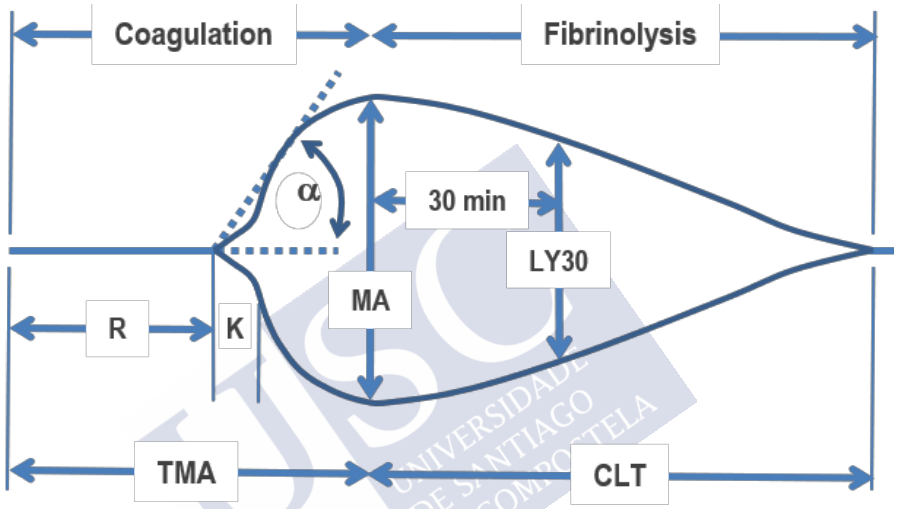

FIGURE 6. TEG tracing and parameters. The TEG R-time and TEG K-time represent the enzymatic and kinetic portion of coagulation, respectively. The TEG angle alpha is related to the fibrinogen concentration and the rapidity of fibrin formation and cross linking, also related to the kinetics of clot formation. The TEG MA or strength of the fibrin clot represents primarily the contribution of fibrin and platelet aggregation to clot formation. TEG LY30 represents percent lysis at 30 minutes after reaching MA. Source: modified from *Measure all Phases of Hemostasis in Whole Blood* in: http://teg.haemonetics.com/en-gb

In the rotational thromboelastometry system (ROTEM), a cylindrical cup containing 340 µl whole blood sample remains fixed while a pin suspended on a ball bearing mechanism initially oscillates through 4° 75′ every 6 seconds through application of a constant force. As the viscoelastic strength of the clot increases, the rotation of the pin is impeded and is detected optically by an image sensor system.[72] ROTEM and TEG provide essentially the same information on clot

kinetics and strength, but because of differences in operating characteristics, the results are not interchangeable.[72]

TEG and ROTEM are subject to unique pre-analytical (e.g., operator induced, such as mix reagents, pipetting) and analytical artifacts (e.g., vibration, pin slippage) that can significantly impact results. Some reports pointed to excessive Coefficient of Variations (CV) for the TEG parameters and the importance of following manufacturer's recommendation that each institution should determine its own normal values before adopting TEG.[73-75] In this regard, a veterinary group of specialists commanded a series of five studies aiming to identify knowledge gaps and to prove the evidence of viscoelastic testing.[76-80] Each study consisted of a comparative review of TEG veterinary literature on five different subjects that brought respective conclusions:

I) Comparability between the TEG and the ROTEM viscoelastic point-of-care instruments. Conclusion: Results from the two analyzers are not directly comparable and extrapolation of the results from one machine to the other should be avoided.[80]

II) Sample acquisition and handling for the TEG and ROTEM. Conclusion: Consistent technique is important for serial sampling, and standardized sampling protocols are recommended. There is insufficient evidence to recommend use of a specific blood collection system, although use of evacuated blood tubes and 21-Ga or larger needles is suggested. Use of 3.2% buffered sodium citrate in a strict 1:9 ratio of citrate to blood is suggested. Suggested tube draw order is discard/serum, followed by citrate, EDTA, and then heparin. Samples should be held at room temperature for 30 minutes prior to analysis.[78]

III) Activating agents and test protocols for the TEG and ROTEM. Conclusion: Overall, assays using citrated samples that employ an activator have significantly lower inherent variability than those that use recalcification alone. There is no evidence to suggest that any one activating agent is superior to another. Results obtained using different activators are not directly comparable. As such, use of more than one assay for complete TEG evaluation of

a patient's coagulation system may be warranted. Standardization of the concentrations of activators would be beneficial.[77]

IV) Definitions and reporting of data for viscoelastic testing in veterinary medicine. Conclusions: All 4 standard TEG and ROTEM variables should be universally reported, and the reporting of shear elastic modulus in addition to MA is encouraged. In reporting data relating to fibrinolysis, the TEG variables LY30, LY60, CL30, CL60, and the ROTEM variables LI30, LI60, ML, LOT, and LT should be documented. Individual studies should report sufficient data on patients and institutional controls to enable definitions of hypo- and hypercoagulability, hyper- and hypofibrinolysis to be evaluated *post-hoc*, and it is recommended that all studies specifically report how these conditions were defined.[79]

V) Nontraditional uses of viscoelastic coagulation monitoring in veterinary species. Conclusions: Nontraditional assays identified included thromboelastography (TEG)-Platelet Mapping (PM), functional fibrinogen assessment, and rapid-TEG (r-TEG). Direct veterinary evidence was found for only the ADP-activated PM, which appears to generate valid data in dogs but not cats or horses. Arachidonic acid activated PM shows high variability and requires further assessment and validation in veterinary species. Functional fibrinogen assays may be performed in veterinary species but may require modification due to species differences in response to abciximab. While TF-activated TEG has been well described in the veterinary literature, the specific r-TEG assay has not been assessed.[76]

Most of the reliability and repeatability issues were solved in the newest and updated version of the TEG technology (i.e., TEG 6s) launched in mid-2017.[81]

Recent publications highlight the utility of TEG to predict tumor histology, stage of the disease and resectability of the tumor in patients undergoing pancreatectomy, and to identify patients with prostate cancer at risk for thrombosis.[82,83] In the veterinary field, there is a consensus today on considering TEG as a sensitive tool to detect

coagulation abnormalities in dogs with a variety of malignancies and for measuring hypercoagulability and hypocoagulability in dogs. [84,85,63,86] The popularity of the test in veterinary medicine is growing and as for the time of publishing this PhD thesis, a PubMed search with in title combined terms of thromboelastography AND canine (7), horse (2), equine (2) and dogs (28) revealed a total of 39 indexed publications. Extending to all fields of search combined terms of thromboelastography AND canine (205), horse (41), equine (44) and dogs (202) for 492 indexed publications. When using the single term of thromboelastography in all fields of search, the PubMed search showed a total of 4,120 indexed publications.

1.3.1 What are the clinical settings that focused our thromboelastography study?

In human beings, approximately 55% of postoperative complications are related to bleeding or thrombotic events; postoperative thromboembolism is more common than bleeding. [2,87,88] While perioperative non-surgical bleeding complications have been well described in human beings in association with specific surgical procedures (i.e., cardiac bypass, liver transplant) as well as in trauma cases, few reports have been published in veterinary medicine.[89-92] It has been recently demonstrated that 26% of Retired Racing Greyhounds (RRG) had excessive hemorrhage 24 h to 48 h after routine gonadectomy. This differs from other breeds of dogs, where the prevalence of bleeding after ovariohysterectomy (OHE) or orchiectomy ranges from 0-2%.[90,93-95] This high prevalence of delayed bleeding after surgical procedures in RRG has been also observed in a pilot study where 10/28 RRG that underwent limb amputation for bone cancer had postoperative hemorrhage severely enough to require transfusion of blood components.[96] Preoperative hemostasis profiles, platelet function analysis, vWF concentration and functional assays test were not able to identify a hemostatic disorder in RRG bleeders' nor to predict which RRG would bleed after surgery.[90] The vast majority of Greyhounds that complete their racing careers are sexually intact and will be spayed or neutered at the time of adoption. Lord and others calculated that spay and neuter before adoption represents at least 15,000 surgeries a year.[97] Considering that a routine spay or neuter in a RRG could result in

hemostatic complications leading to readmission to the clinic, and in some cases transfusion of blood or blood components, identifying the dogs at risk, or developing a simple protocol for prevention of this complication will be extremely valuable.

The nexus between venous thrombosis and malignancy in people was first described by Trousseau (Trosseau's syndrome) in the late 1800s.[98] Most recently, several studies in humans have shown a strong association between idiopathic venous thrombosis and subsequent development of clinical cancer; in addition, patients with malignancy have a higher risk, an incidence up to 40% higher, than the general population to develop postoperative thromboembolic complications.[99,100] Depending on the tumor type, the prevalence of thrombosis in an autopsy series of patients with cancer was as high as 30%.[99,100] In addition to thrombotic disorders, DIC has been reported in as many as 62% of human patients with malignant solid tumors, and it is relatively common in dogs.[101]

In veterinary medicine, the tests routinely used for evaluation of hemostasis, such as PT and aPTT, rarely detect hypercoagulable states.[102] Identifying dogs on prothrombotic state to establish treatments that reduce the risk of thrombosis, and to determine if the use of anticoagulants/blood thinners (i.e., heparin, acetylsalicylic acid) in hypercoagulable dogs diagnosed with cancer has an effect on the biological behavior of the malignancy, could be of clinical significance.

Hemostatic changes are commonly observed in horses with sepsis or gastrointestinal disorders.[103-105] In these pathologic conditions, blood vessels, platelets, clotting factors, and/or fibrinolysis can be altered, resulting in a hypo- or hypercoagulable state. Horses with colic or colitis frequently develop hemostatic dysfunction resulting in secondary complications such as DIC and thrombophlebitis.[103-109] It has been reported that 32% of hospitalized horses with colitis have subclinical DIC based on three or more of the following abnormalities: thrombocytopenia, prolonged PT and aPTT, increased fibrin degradation products (FDP), decreased fibrinogen concentration, and decreased AT activity.[109] Furthermore, in the same study, a horse with acute colitis was 8 times more likely to die or be euthanatized if a

diagnosis of clinical DIC was made.[109] A recent study has shown that horses with severe gastrointestinal disorders have widespread intravascular fibrin deposits that are consistent with capillary microthrombosis, multiorgan failure, and DIC.[110] It could be extremely helpful if TEG would identify horses at risk to develop DIC that may result in fibrin deposit in the microvasculature of the organs and multiple organ failure. In that scenario, it would be possible to establish early therapies and more accurate prognosis.

2. OBJECTIVES

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2. OBJECTIVES

The general aim of this PhD project was to evaluate the clinical-pathologic value of the TEG for the diagnosis of common hemostatic abnormalities in domestic animals. Our specific objectives were:

1. Evaluate hemostasis in healthy RRG by means of TEG and to do a comparative analysis with healthy non-Greyhound dogs.
2. Characterize the perioperative hemostatic singularities in RRG.
    a. Identify TEG parameters that would predict bleeding and/or other hemostatic abnormalities in RRG undergoing surgical procedures.
3. Evaluate clot formation in dogs with carcinomas using common tests for hemostasis (i.e., PT, aPTT, fibrinogen concentration and PLT count) and TEG.
    a. Disclose the hemostatic abnormalities associated with carcinoma in dogs.
4. Investigate the diagnostic value of TEG for the detection of DIC in a dog.
5. Determine the diagnostic and prognostic value of TEG in horses suffering from common diseases frequently associated with coagulopathies by correlating:
    a. Detection of coagulopathies (e.g., DIC) by common test for hemostasis and TEG.
    b. Detection of fibrin deposits by histological (i.e., H&E and PTAH) and immunohistochemical analysis.

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3. MATERIAL & METHODS

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3. MATERIAL & METHODS

3.1 ANIMALS INCLUDED IN THE STUDIES

3.1.1 Dogs

3.1.1.1 Healthy Dogs - Control Groups

A total of thirty-four healthy non-RRG dogs were included in the studies. Of them, fifteen were enrolled in the TEG study in RRG, and nineteen in the TEG study in dogs with carcinoma. Enrolled non-RRG healthy dogs had no previous history of bleeding disorders and no therapies that could affect hemostasis. All dogs were considered healthy based on absence of clinical signs of illness, results of physical examination (PE), complete blood count (CBC) and hemostasis panel (aPTT, PT, fibrinogen). Mix breed dog was the most representative breed.

3.1.1.2 Retired Racing Greyhounds

Forty-nine RRG were included in the studies. Of them, twenty-eight were enrolled in the TEG study in RRG, and twenty-one in the TEG study after gonadectomy. Enrolled RRG had no previous history of bleeding disorders and no abnormalities on PE, CBC, serum biochemistry profiles (in dogs more than 5 years of age), and SNAP test (SNAP-4DX; IDEXX Laboratories) for common vector borne diseases (i.e., *Anaplasma phagocytophilum*, *Ehrlichia canis*, *Dirofilaria immitis* and *Borrelia burgdorferi*).

3.1.1.3 Dogs with Carcinoma

We used thirty-two dogs with carcinomas with no history of receiving or had received anticancer chemotherapy, and/or COX-1/COX-2 inhibitors within 2 weeks of enrollment in the study. The

diagnosis of malignancy was done by histopathology (i.e., punch biopsy), cytology (i.e., fine needle aspirate), or both. Staging of the disease included regional lymph node aspiration cytology or histopathology, abdominal ultrasonography, and/or thoracic radiography (three views).

3.1.1.4 Dog with Hemangiosarcoma

We evaluated a 10 years old castrated male Bichon Frise for hemostatic abnormalities using TEG, aPTT, PT, Anti-Thrombine (AT) and D-dimers. Diagnosis of hemangiosarcoma (HAS) was done based on cytology and histopathology. Staging consisted of full abdominal ultrasound and 3 views thoracic radiographs.

3.1.2 Horses

We included in the study 28 horses with TEG test done less than 24 h before death, and necropsy performed no later than 24 h after death. Horses died because of progressive disease or euthanized based on poor prognosis or animal control.

3.1.2.1 Healthy Horses – Control Group

With the aim to statistically power the correlation study, we included five horses considered healthy based on PE and no previous history of coagulation abnormalities.

3.1.2.2 Horses with Possible Hemostatic Abnormalities

A total of 23 horses with disorders frequently associated with coagulopathies were enrolled in the study. Of them, six horses were diagnosed with a gastrointestinal disorder (i.e., colic syndrome), 2 with colitis (i.e., salmonellosis), 5 with neuro-orthopedic disease (i.e., laminitis, navicular disease, Wobbler syndrome), 6 with non-gastrointestinal inflammatory disease (i.e., septicemia, pneumonia, strangles, Potomac fever), and one horse in each case for squamous cell carcinoma, hepatic amyloidosis, splenomegaly and Equine Protozoal Myeloencephalitis (EPM).

3.2 Ethical And Owner's Consent Approval

Animals included in the studies were enrolled after signed owner consent. The use of client-owned animals was approved by the Veterinary Teaching Hospital Board (Ohio State University, OSU).

3.3 Blood, Plasma And Serum Samples

In dogs, blood samples were collected by jugular venipuncture using a 21 gauge needle and a 6 mL syringe. Blood samples were placed into collection tubes (2.7 mL, 3.2% buffered sodium citrate [0.3 mL; 0.109 M] 1:9 anticoagulant/blood ratio, Vacutainer BD), mixed gently, and stored for 30-45 min at room temperature. Citrated tubes were centrifuged (1,380 g for 10 min) within 45 min of sampling to obtain plasma for hemostasis assays. Remaining of canine plasma samples was stored at -30 ºC until evaluated for Thrombin Anti-Thrombin (TAT) and PAI-1 immunoassays. Serum samples obtained from blood samples were placed into a 3 mL red top serum separator tube (Monoject; Mansfield, MA), rested and centrifuged (Allega X-30) at 3000 rpm for 10 min.

In horses, blood samples were collected by jugular venipuncture using a 20 gauge multi sample blood collection needle (Vacutainer BD). Blood samples were placed into collection tubes (4.5 mL, 3.2% buffered sodium citrate, 1:9 anticoagulant/blood ratio, Vacutainer BD) and mixed gently. One citrated tube was stored for 30 to 60 minutes at room temperature in a tube rack for TEG analysis. The second citrated tube was centrifuged (1,380 g for 10 min) within 45 min of sampling to obtain plasma for hemostasis assays.

3.4 Complete Blood Counts, Biochemistry Profiles And SNAP Test

CBC were done with 0.8 mL of citrated blood in flow cytometer hematology analyzers (LaserCyte; IDEXX Laboratories, Westbrook, ME) and/or in a Cell-Dyn 3500 cytometer, Abbott Laboratories). About 3 drops of pipetted citrated blood were used for SNAP (SNAP-4DX; IDEXX Laboratories, Westbrook, ME) test for common vector borne

diseases in dogs. Finally, about 0.5-1 mL of serum was used to perform biochemistry profiles (COBAS c501 chemistry analyzer (Roche Diagnostics, Indianapolis, IN). The laboratory reference ranges established by the Institutional Veterinary Clinical Pathology Laboratory (OSU) for healthy dogs and horses were used to define hemostatic abnormalities (i.e., thrombocytosis in dogs if PLT > 424 $x10^9$ U/L) found on CBC, biochemistry profile and hemostatic profile (i.e. PT, aPTT, fibrinogen).

3.5 TAT, PAI-1 AND COAGULATION PROFILE

TAT (Enzygnost TAT micro, Dade Behring Marburg) and PAI-1 (Spectrolyse/pL PAI, Trinity Biotech) activities were determined in canine plasma by enzyme immunoassay kits according to the manufacturer's instructions. TAT assays were performed at the Animal Health Diagnostic Center, Cornell University, NY, and were run in duplicate after a single thaw and the average of those values was used for statistical comparison. TAT average concentration of the human control plasma provided by the manufacturer to assure methodology accuracy was 9.9 lg/L (range: 7.2–11.9 lg/L).

A single PAI-1 determination after single thaw was obtained per dog at the Clinical Trials Laboratory of the Department of Veterinary Biosciences, OSU. Therefore, PAI-1 CVi is unknown. The results obtained from 15 healthy dogs that were evaluated previously using the same methodology were used as reference control range for a healthy population (data not shown). The reference ranges used to define hemostatic abnormalities on TAT and PAI were established based on the results obtained from the control group (i.e., mean ± 2SD).

PT and aPTT coagulation curves for dogs and horses were obtained by nephelometry using an ACL-200 analyzer (ACL-200 Automated Coagulation Laboratory, Instrumentation Laboratory) and commercial reagents (Dade Actin FS, Dade Behring for aPTT measurements; thromboplastin Li, Helena Diagnostics for PT measurements). Plasma fibrinogen concentration was calculated as a parameter derived from the

PT coagulation curve. In addition, pooled plasma samples from 3 non-RRG healthy dogs were used as control group.

Due to a lack of plasma samples, it was not possible to do hemostasis profile analysis (i.e., PT, aPTT, fibrinogen) in four dogs of each group (control and RRG). Also, only 21 carcinoma dogs and 15 controls were included for TAT and PAI-1 evaluation.

3.6 Surgical Procedure In Dogs– Gonadectomies & Anesthetic Protocol

Gonadectomies in female dogs consisted of a 3-clamp technique with a 10 to 15 cm midline incision through the skin and subcutaneous tissues, from 1 cm caudal to the umbilicus to the level of the most caudally located mammary glands; for male dogs, a three-clamp technique with small pre-scrotal incision through the skin and subcutaneous tissues for closed castration. All gonadectomies and anesthetic events were supervised by Diplomates of the American College of Veterinary Surgery and Diplomates of the American College of Anesthesia respectively.

Pre-anesthetic protocol consisted of 0.05 mg/kg buprenorphine (Buprenorphine HCl; Bedford Laboratories) and 0.05 mg/kg acepromazine (Aceproject; Butler Animal Health Supply) intramuscularly in addition to a prophylactic dose (22 mg/kg) of intravenous cefazolin sodium (Cephazolin sodium; Sandoz). Induction of general anesthesia was accomplished with 5 mg/kg ketamine (Ketaset; Fort Dodge Animal Health) and 0.25 mg/kg diazepam (Diazepam; Hospira) intravenously and maintained using isoflurane (Isosol; Vedco) in 100% oxygen. Respiration was supported with intermittent positive-pressure ventilation and intraoperative fluid therapy with lactated Ringer's solution (10 ml/kg/h intravenously) (Ringer Lactate Solution; Baxter Healthcare Corporation). Postoperative analgesia consisted of a single intramuscular injection of carprofen (4 mg/kg) (Rimadyl; Pfizer). All dogs were kept at the hospital for a minimum of four days and underwent daily physical examinations.

3.7 BLEEDING SCORE IN RETIRED RACING GREYHOUNDS

The magnitude of the bleeding in each RRG was estimated immediately after gonadectomy, 24 h and 48 h after surgery using a bleeding scoring system adapted from that proposed by Buchanan and Adix for children with idiopathic thrombocytopenic purpura and recently validated in RRG.[90] RRG with a bleeding score of greater than or equal to 2 at 24 to 48 h after surgery were designated as "bleeders", whereas those with a bleeding score of less than 2 at 24 to 48 h after surgery were designated as "non-bleeders". The bleeding score system is shown in Table 1.

Table 1. Bleeding score system used to classify the bleeding episodes post-gonadectomy in RRG.

Score	
0	No new bleeding
1	Questionable new petechiae or bruising
2	New cutaneous and/or mucosal hemorrhagic lesions
3	Moderate to severe cutaneous or mucosal bleeding without measurable decline in HCT
4	Severe external bleeding of sufficient magnitude to decrease HCT by ≥6% points

3.8 HISTOLOGICAL EXAMINATION, IMMUNOHISTOCHEMISTRY AND FIBRIN DEPOSIT SCORING IN HORSES

Tissue samples from kidney, liver and lung of horses were fixed in Bouin's solution for at least 24 h, embedded in paraffin wax, sectioned at 2-3 μm in thickness, and stained with hematoxylin and eosin (H&E) and phosphotungstic acid hematoxylin (PTAH) by standard procedures for light microscopy. For immunohistochemistry (IHC), unless otherwise stated, all incubations described here were done at room temperature in a humid chamber with an automated stainer (Dako Autostainer, Dako). Sections were passed through graded alcohols to phosphate-buffered saline solution (PBS) (pH 7.4, 0.01 M). Washing procedures consisted of 3 min incubation in PBS. Endogenous peroxidase activity was blocked by incubation in Peroxidase Blocking Reagent (Dako, Denmark) for 90 min, and after a rinse in PBS, antigens

were treated with 0.5% bovine serum albumin for 15 min to block non-specific antibody binding, rinsed again with PBS and incubated with antibody against fibrinogen/fibrin (Dako, Denmark) at a dilution 1:2500 for 30 min. After washing, slides were incubated with polyclonal anti-rabbit EnVision System Labelled Polymer-horseradish peroxidase (DakoCytomation, CA) for 30 min. After further rinsing, the sections were finally developed using 3,3-diaminobenzidine (DakoCytomation, CA), immersed in deionized water to stop the reaction, counterstained with hematoxylin, dehydrated, and coverslipped. In each series of stained sections, positive and negative controls were included to assess the specificity of the assay. Sections of fresh blood clot were used as positive controls. Negative control slides were sections in which the primary antibody was replaced by PBS.

The presence of lesions and microvascular fibrin deposits in horses was recorded blindly by a Diplomate of the American College of Veterinary Pathology for each sample stained with H&E, PTAH and IHC. The amount of fibrin detected by PTAH and IHC in kidney, lung and liver sections was scored from 0 to 4 using a modified scoring system from a previously reported study and as shown in Table 2.[11]

Table 2. Score system used to classify the amount of fibrin deposits in sections of kidney, liver and lung of horses by means of phosphotungstic acid hematoxylin (PTAH) staining and immunohistochemistry (IHC) against fibrinogen/fibrin.

Score	Kidney	Liver	Lung
0	Absent staining or positive in 10% of the glomeruli	Absent staining or 10% positive fields	Absent staining or 10% positive fields
1	Staining in glomerular capillaries in 11–25% of glomeruli in nonconsecutive fields	11–25% positive fields with staining in hepatic sinusoids in nonconsecutive fields	11–25% positive fields with staining in alveolar capillaries in nonconsecutive fields
2	Staining in glomerular capillaries in 26–50% of glomeruli	26–50% positive fields with staining in hepatic sinusoids in half of the fields in an almost continuous staining pattern	26–50% positive fields with staining in alveolar capillaries in half of the fields in an almost continuous staining pattern
3	Staining in glomerular capillaries in 51–75% of glomeruli	51–75% positive fields with staining in hepatic sinusoids in 50% of the fields in a continuous staining pattern	51–75% positive fields with staining in alveolar capillaries In 50% of the fields in a continuous staining pattern
4	Staining in glomerular capillaries in >75% of glomeruli	>76% positive fields with staining in hepatic sinusoids in a continuous staining pattern with a fibrillar meshwork	>76% positive fields with staining in alveolar capillaries in a continuous staining pattern with a fibrillar meshwork

To compare both stains for clinical assessment, each horse was meta-scored from 0 to 4 according to the amount of fibrin thrombi observed in its tissue samples, as previously described, and as shown in Table 3.[110]

Table 3. Meta-score system used to categorize horses according to the amount of fibrin deposits in organs established in Table 1.

Score	
0	Horses not classified as 1, 2, 3 or 4
1	At least 2 tissue samples scored 1 and no tissue samples of higher score
2	At least 1 tissue sample scored 2 and no tissue samples with higher scores
3	At least 1 tissue sample scored 3 and no samples with higher scores
4	At least 1 tissue sample with a 4 score

3.9 THROMBOELASTOGRAPHIC ANALYSIS

A single TEG test was started 30–45 min after sampling using blood obtained from the collected tubes. For dogs undergoing gonadectomies, a TEG test was done in each dog before surgery (before premedication) and 24 h after surgery. In horses, a TEG test was done no less than 24 h before death. TEG test were done by the same operator (PVS).

Initially, 20 mL of $CaCl_2$ was placed in the prewarmed cup of the TEG-5000; then, 340 mL of citrated blood was added for a total volume of 360 mL. Tracings were obtained after 120–180 min of running time at 37 ºC. Routine quality controls with normocoagulable and hypocoagulable tracings (level I and level II) and e-test controls were run in each TEG channel before each sampling test to achieve the recommended quality assurance.[63] The reference ranges used to define hemostatic abnormalities on TEG were established based on the results obtained from the control group (i.e., mean ± 2SD). As for example, hypercoagulability was defined and diagnosed as TEG_{TG} > 2SD of the mean for healthy control dogs.

3.10 STATISTICAL ANALYSIS

All data were evaluated for normality by the D'Agostino and/or Kolmogorov-Smirnov test. Statistical analysis was performed using a Student's test for normally distributed data, and a Mann-Whitney U for data with non-Gaussian distribution. One-way ANOVA for evaluation of three or more independent groups (e.g., metastases and multiple hemostatic parameters). Fisher's exact test for categorical data was used to evaluate differences such as gender. Cohen's kappa coefficient and McNemar exact test to evaluate the significance of agreement concerning the presence of fibrin deposits and TEG–IHC. Pearson's and Spearman's rank correlation coefficients were used for correlation analysis of numerical data as for example HCT, APTT, TEG_R, TEG_K, TEG_{angle}, TEG_{MA}, TEG_G, and TEG_{LY60}. Correlation was categorized as excellent (0.93–0.99), good (0.80–0.92), fair (0.6– 0.79), or poor (<0.59).[111] Statistical significance was set at $P < 0.05$. A commercial statistical software package (Prism version 4.0, GraphPad Software) was used to analyze the data.

4. RESULTS

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4. RESULTS

4.1 THROMBOELASTOGRAPHY IN RETIRED RACING GREYHOUNDS

For this study, we sampled 43 dogs (28 RRG and 15 non-Greyhounds). In the study group (RRG), there were 11 intact females and 7 intact males, and 2 spayed females and 8 castrated males. The mean age was 5.6 years (range 3–9 years). In the non-RRG group, there were 2 intact females and 1 an intact male, 4 were spayed females and 8 castrated males. The mean age was 4.5 years (range 2–14 years).

Statistic evaluation revealed significant differences between RRG and non-Greyhounds for aPTT, hematocrit (HCT), PLT count and for the following TEG parameters: TEG_K, TEG_{angle}, TEG_{MA} and TEG_G as shown in Table 4 & Figure 7. In RRG, clotting kinetics were slower and clot strength weaker than in non-RRG, as represented in Figure 8, supporting the increased tendency to bleed observed after minor trauma or surgical procedures in the breed. Higher HCT and low PLT count, a well-known hematologic characteristic of Greyhounds, talks about their unique rheological properties.

Table 4. Non-Greyhounds vs. Greyhounds TEG and coagulation panel results.

TEG & Hemo Panel	Non-Greyhounds Mean ± SD (range) n= 15	Greyhounds Mean ± SD (range) n= 28	P value	Control Mean ± SD n=3
R-time (minutes)	3.7 ± 1.6 (1.7–8.2)	4.3 ± 1.7 (1.8–8.8)	.247	
*K-time (minutes)	2.5 ± 0.9 (1.3–4.5)	3.8 ± 1.4 (1.8–7.7)	.002	
*Angle (degrees)	59.8 ± 7.0 (46.3–71.4)	50.0 ± 8.0 (34.3–64.9)	.000	
*MA (mm)	53.1 ± 5.6 (43.5–61.0)	47.6 ± 5.6 (38.0–60.9)	.003	
*G (dyn/cm^2)	5811±1256(3843–7810)	4647±1097(3058–7772)	.003	
LY60 (%)	3.1 ± 2.5 (0.0–8.6)	2.8 ± 5.0 (0.0–19.2)	.080	
*PLT (10^9 U/L)	257 ± 88 (142–421)	200 ± 49 (105–314)	.010	
*HCT (%)	41.81 ± 4.3 (35.0–48.4)	47.61 ± 5.1 (38.0–59.0)	.000	
PT(seconds)	6.9 ± 0.5 (6.1–8)	6.7 ± 0.8 (6.1–7.9)	.106	7.0±0.5
*aPTT (seconds)	12.17 ± 0.9 (9.9–14)	13.3 ± 1.15(11.1–15.7)	.002	10.4±0.8
Fib (mg/dL)	153.8 ± 51.3(102–278)	173.7 ± 97(73–365)	.464	99.3±35.4

The asterisks denote statistically significant differences between non-Greyhounds and Greyhounds. TEG: Thromboelastography; R-time: Reaction time; K-time: clot kinetics; Angle: angle of the TEG trace; MA: Maximum Amplitude; G value: shear modulus strength; LY60: percent lysis at 60 minutes after reaching MA; aPTT: activated Partial Thromboplastin Time; Fib: Fibrinogen; HCT: Hematocrit; n: number of dogs; PLT: Platelet count; PT: Prothrombin Time; SD: Standard Deviation.

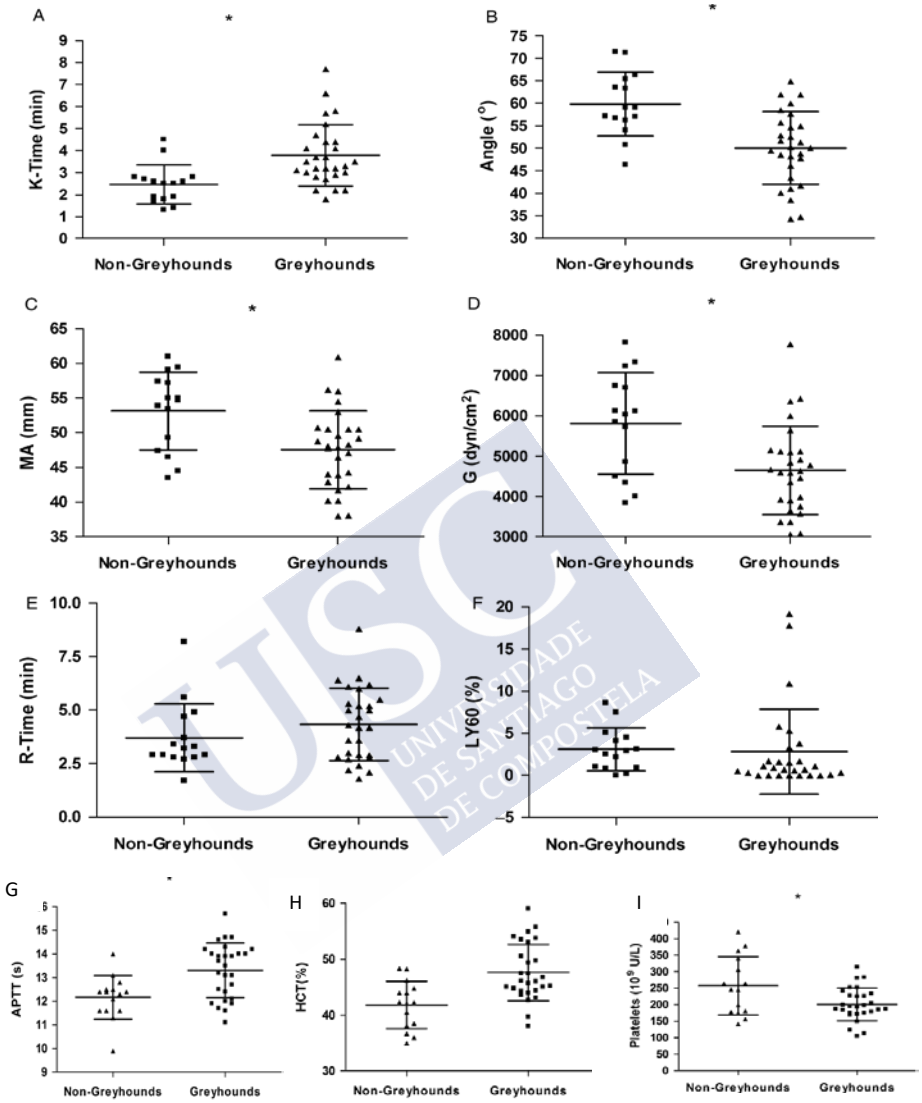

FIGURE 7. Vertical scatter plots showing the difference between the means for K-time: clot kinetics (A); Angle: angle of the TEG trace (B); MA: Maximum Amplitude (C); G-value: shear modulus strength (D); R-time: Reaction time (E); LY60: percent lysis at 60 minutes after reaching MA (F); APTT: Activated Partial Thromboplastin Time (G); HCT: hematocrit, and platelet count (I) for non-Greyhounds vs. Greyhounds. Horizontal bars indicate the Mean ± Standard Deviation for each group, and the asterisk marks plots with statistically significant differences between the groups.

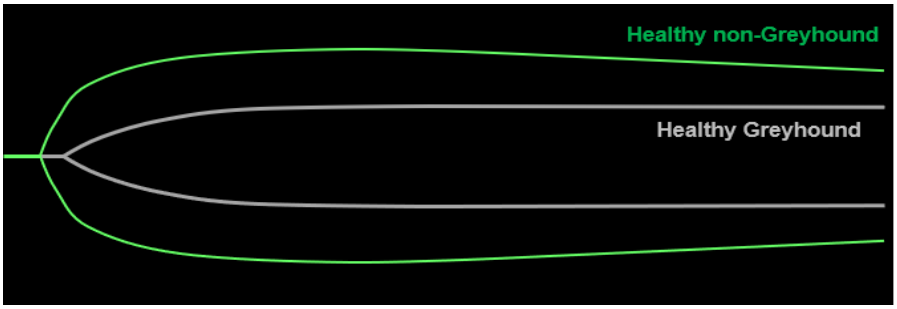

FIGURE 8. Representative superposed TEG tracings of a healthy non-Greyhound (outside tracing, green) and a healthy Greyhound (inside tracing, white).

4.2 THROMBOELASTOGRAPHY AFTER GONADECTOMY IN RETIRED RACING GREYHOUNDS

Twenty-one healthy RRG were gonadectomized using surgical procedures and standardized protocols for general anesthesia described before. TEG test were done pre-surgery and 24 h post-surgery. The median age was 5 years (range 2–9 years). There were 11 intact females and 10 intact males, and the female:male ratio was 1.1:1.

The dogs were categorized as "bleeders", 8 dogs, or "non-bleeders", 13 dogs, based on the bleeding scoring system presented in Table 3 of the Material & Methods section. An example of a "bleeder" RRG after gonadectomy is shown in Figure 9.

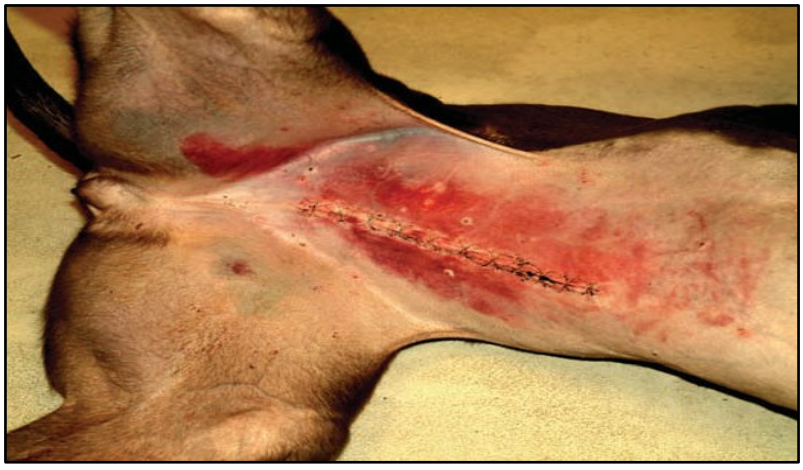

FIGURE 9. Postoperative bleeding in a RRG 24 h after surgery.

There were no significant differences pre-operatively between "bleeders" and "non-bleeders" for TEG parameters, aPTT, fibrinogen concentration, PLT count, or hemoglobin (HGB) concentration as shown in Table 5. However, increases in TEG_{MA} and TEG_G observed in the non-bleeding RRG suggest the formation of a stronger clot in this group.

Table 5. RRG bleeders vs. non-bleeders TEG and coagulation panel results before and after gonadectomy.

Variables	Non bleeders before surgery median (range) n=13	Bleeders before surgery median (range) n=8	Non bleeders 24 h after surgery median (range) n=13	Bleeders 24 h after surgery median (range) n=8	Control Mean ± SD Reference range
R (minutes)	4.7 (1.8-7.2)	2.9 (2.2-8.8)	4.5 (2.8-12.6)	10.2 (5.0-24.2)	(1.6-8.2)
K (minutes)	3.7 (2.2-5.8)	3.2 (1.8-4.7)	2.1 (1.5-8.3)	5.2 (2.4-11.8)	(1.1-7.1)
Angle (degrees)	49.5 (39.9-56.0)	52.2 (41.0-64.9)	60.5 (25.6-66.3)	38.3 (16.8-56.2)	(33.3-63.3)
MA (mm)	48.8 (39.0-56.6)	48.5 (42.2-60.9)	59.8 (39.1-68.0)	51.6 (49.9-57.8)	(35.7-56.5)
G (dyn/cm2)	4771 (3192-6358)	4719 (3653-7772)	7451 (3217-10,614)	5339 (3906-6837)	(2515-6227)
LY60 (%)	1.6 (0.0-19.2)	2.2 (0.0-5.8)	0.5 (0.0-4.7)	0.5 (0.5-1.7)	(0.0-4.4)
PT (seconds)	6.8 (6.5-7.5)	6.9 (6.1-7.9)	7.2 (6.6-8.2)	7.1 (6.3-7.6)	7.3 ± (0.5)
aPTT (seconds)	12.6 (11.1-14.6)	12.1 (13.5-14.5)	10.9 (8.9-13.5)	12.3 (10.4-13.6)	10.8± (0.7)
Fib (mg/dl)	184 (81-339)	198 (93-365)	285 (165-412)	265 (161-358)	136 ± (42)
PLT count (109 U/L)	213.3 (113-314)	202.1 (105-252)	–	–	(106-424)
Hgb (g/dl)	17.5 (15-21.2)	16.7 (15.7-18.4)	–	–	(11.9-18.4)

TEG parameters: R-time: Reaction time; K-time: clot kinetics; Angle: angle of the TEG trace; MA: Maximum Amplitude; G value: shear modulus strength; LY60: percent lysis at 60 minutes after reaching MA; aPTT: activated Partial Thromboplastin Time; Fib: Fibrinogen; Hgb: Hemoglobin; PLT: Platelet count; PT: Prothrombin Time. On the last right column, TEG reference ranges for healthy greyhounds and control plasma test group.

Afterwards, we decided to analyze statistically all the parameters as possible predictors of the bleeding episodes. The analysis of the HCT and PLT count revealed no effect on the outcome, and the same was true for the variables that evaluated clot kinetics, fibrinolysis (i.e. aPTT, PT, TEG_R, TEG_K, TEG_{LY60}), or any of the plasma based coagulation analyzer test used in this study (i.e. aPTT, PT, fibrinogen). Only the TEG variables that represent the fibrin cross-linking of the clot (i.e. TEG_{angle}) and the fibrin to platelet interaction or the strength of the clot (i.e. TEG_{MA} and TEG_G) were considered predictors of the outcome. These results are shown in Table 6.

Table 6. TEG and coagulation panel parameters evaluated as possible predictors of bleeding episodes in RRG.

Variable	OR	CI-95%	P-value	Mean (SD) difference	Mean (SD)	F:M
R (minutes)	1.269	0.982-1.639	0.06	3.64 (5.72)		
K (minutes)	1.696	0.983-2.92	0.05	0.45 (3.0)		
Angle (degrees)	0.903	0.823-0.991	0.03	–2.5 (16.7)		
MA (mm)	0.833	0.705-0.985	0.03	6.0 (8.25)		
G (dyn/cm2)	0.999	0.998-1.000	0.02	1502.6(2034.4)		
LY60 (%)	1.028	0.810-1.301	0.81	–2.34 (3.9)		
PT (seconds)	1.337	0.405-4.421	0.63	–0.33 (0.75)		
aPTT (seconds)	0.781	0.367-1.660	0.51	1.43 (1.20)		
Fib (mg/dl)	1.001	0.995-1.008	0.67	–82.15 (136.2)		
Age (years)	0.949	0.575-1.568	0.83		5.35(1.84)	
HCT (%)	0.907	0.724-1.136	0.39		47.4(4.2)	
PLT count (U/µl)	0.996	0.980-1.013	0.65		208.85(55.3)	
Gender(F:M)	0.148	0.020-1.081	0.05			1.1:1

TEG parameters: R-time: Reaction time; K-time: clot kinetics; Angle: angle of the TEG trace; MA: Maximum Amplitude; G value: shear modulus strength; LY60: percent lysis at 60 minutes after reaching MA; aPTT: activated Partial Thromboplastin Time; CI: Confidence Interval; Fib: Fibrinogen; Gender ratio: F:M female/male; HCT: Hematocrit; Mean difference: Mean difference before and after gonadectomy; OR: Odds Ratio; PLT: Platelet count; PT: Prothrombin Time; SD: Standard Deviation.

Variation in TEG parameters were clearly revealed between bleeders and non-bleeders when postoperative TEG parameters were compared to pre-surgery baseline and expressed as a percentage (positive if they increased, and negative if they decreased). In the non-bleeders, there were virtually no changes for TEG_R (median before vs. after surgery: 4.7 vs. 4.5 min); however, there was a ‑43% change in TEG_K (median before vs. after surgery: 3.7 vs. 2.1 min), a +20% in both TEG_{angle} (median before vs. after surgery: 49.5 vs. 60.5 degrees) and TEG_{MA} (median before vs. after surgery: 48.8 vs. 59.8 mm), and +58% in TEG_G (median before vs. after surgery: 4771 vs. 7451 dyn/ cm^2), supporting an increase in clot strength. In contrast, in the bleeders, there was a +251% prolongation in TEG_R (median before vs. after surgery: 2.9 vs. 10.2 min), and +62% change in TEG_K (median before vs. after surgery: 3.2 vs. 5.2 min), a -27% change in TEG_{angle} (median before vs. after surgery: 52.2 vs. 38.3 degrees), a +6% change in TEG_{MA} (median before vs. after surgery: 48.5 vs. 51.6 mm) and +13% change in TEG_G (median before vs. after surgery: 4719 vs. 5339 dyn/cm^2), supporting slower clot kinetics and reduced capacity to build a stronger clot (Figures 10 & 11).

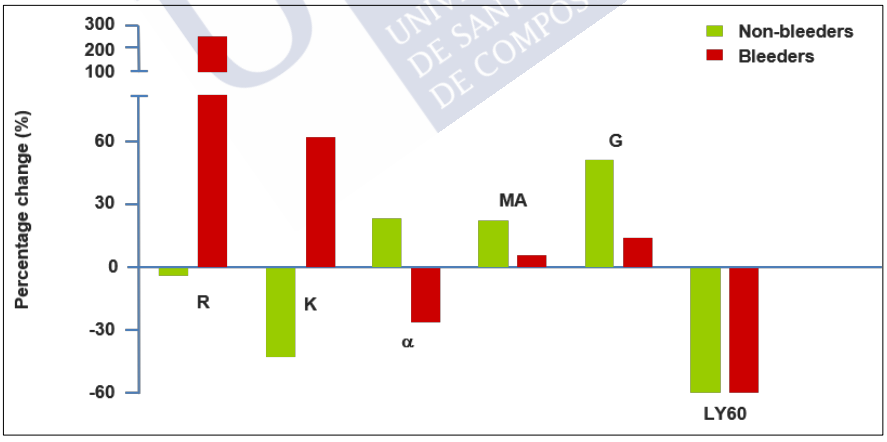

FIGURE 10. Comparative (bleeders vs. non-bleeders) postoperative changes in TEG parameters expressed as a percentage: “positive” if they increased and “negative” if decreased. TEG parameters: R-time: Reaction time; K-time: clot kinetics; Angle: angle of the TEG trace; MA: Maximum Amplitude; G value: shear modulus strength; LY60: percent lysis at 60 minutes after MA.

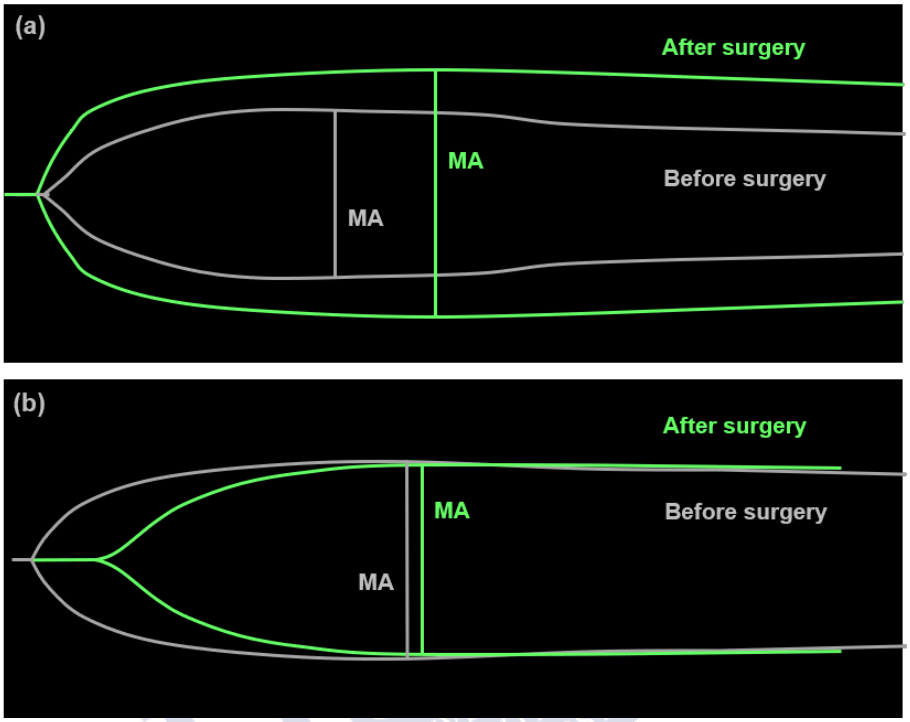

FIGURE 11. (a) Comparative TEG tracings in a "non-bleeder" Greyhound showing the reactive hypercoagulable state with increased TEGMA (Maximum Amplitude) 24 h after surgery (green tracing) and the normocoagulable state before surgery (white tracing). (b) Comparative TEG tracings in a "bleeder" Greyhound showing absence of a reactive hypercoagulable state with no increase for TEGMA (Maximum Amplitude) 24 h after surgery (green tracing) and the normocoagulable state before surgery (white tracing).

4.3 THROMBOELASTOGRAPHY IN DOGS WITH CARCINOMA

Thirty-two dogs with carcinoma (study group) and 19 age-matched healthy dogs (control group), all neutered, were evaluated for hemostatic abnormalities. In the study group, the median age was 9.75 years (range 5–15 years). There were 20 females and 12 males resulting in a female:male ratio of 1.6:1. In the control group the median age was 9.68 years (range 6–13 years). There were 6 females and 13 males resulting in a female:male ratio of 1:2.1. Tumors types included: thyroid (n=9), anal sac (n=7), mammary gland (n=5), transitional cell of the bladder (n=3), pulmonary (n=3), hepatic (n=3), nasal (n=1), and

gastrointestinal (n=1) carcinomas. All carcinoma dogs had thoracic radiograph evaluation, but 10 lacked abdominal ultrasonography; however, 8 of these 10 dogs had thyroid carcinoma and the other two dogs had pulmonary carcinoma, tumors that rarely metastasize to abdominal organs. For 17/32 dogs, there were regional and/or distant metastases.

TEG, hemostasis profile (i.e., PT, aPTT, fibrinogen concentration), PLT count, TAT complexes, and PAI-1 activity studies were conducted. The median for the TAT intra-assay coefficient of variation (CVi) was 5.6% (range: 0.1–23%; 73% of the samples had <10% CVi). A severe inter-individual variability was observed in the healthy dogs (range: 1.2–40 lg/L). One outlier was identified in the control group and excluded from data analysis.

There were no significant differences in age, gender or HCT between the two groups (Tables 7 & 8). Dogs with carcinomas had faster thrombus generation (TEG_{TG} 834.8 ± 91.1 vs. 707.8 29 ± 75.8 mm/min; mean ± SD), increased fibrinogen concentration (276 vs. 151 mg/dL), and PLT count (425 vs. 324 x 10^9 U/L), but decreased PAI-1 activity (15.7 vs. 26.2 IU/mL) as shown in Figure 12 and Tables 8 & 9. The most common hemostatic abnormalities found in carcinoma dogs were: hypercoagulability (TEG_{TG} > mean + 2SD of healthy dogs) in 15 out of 32 (46%), thrombocytosis (PLT > 424 x 10^9 U/L) in 15 out of 32 (46%), and hyperfibrinogenemia (fibrinogen > 384 mg/dl) in 9 out of 32 (28%). DIC was uncommon and extent of disease was not correlated with hypercoagulability. TEG_{TG} showed good correlation with fibrinogen (r=0.80), and hyperfibrinogenemia seems to be a main factor of the hypercoagulable state in carcinoma dogs (Figure 12). No significant differences were observed for TEG parameters and hemostatic panel between carcinoma dogs with metastatic disease and carcinoma dogs with no evidence of metastasis (Table 9).

Table 7. TEG values in healthy and carcinoma dogs.

TEG Results	Healthy Control mean ± SD n=19	Carcinoma mean ± SD n=32	*p-value*
R (min)	3.5±1.6	3.8±1.5	p=0.443
*K (min)	1.9±0.6	1.5±0.5	p=0.012
*Angle (degrees)	64.2±6.8	69.0±6.7	p=0.018
*MA (mm)	58.0±6.0	68.0±7.1	p<0.01
*G (dyn/cm^2)	7157±1746	11432±3827	p<0.01
Gender (f/m) Ratio	1/2.1	1.6/1	p=0.05
Age yr Median (range)	9.68 ± 1.8 (6-13)	9.75 ±2.5 (5-15)	p=0.95

TEG parameters: R: Reaction time; K: clot kinetics; Angle: angle of the TEG trace; MA: Maximum Amplitude; G value: shear modulus strength. f/m: female/male; yr: year; n: number of dogs; SD: Standard Deviation. Asterisk denotes statistically significant difference between groups.

Table 8. Hemostatic parameters in healthy and carcinoma dogs.

Results	HCT % Mean±SD (range)	*PLT Ux10^9/L Median (range)	aPTT (s) Mean±SD (range)	PT (s) Mean±SD (range)	*Fib mg/dl Median (range)	*TEG_{TG} mm/min Mean±SD (range)	TAT µg/L Median (range)	*PAI-1 IU/ml Median (range)
Healthy	43.8±4.6 (33.5-50.2) [n=19]	295 (169-477) [n=19]	11.7±1.2 (8.7-13.4) [n=15]	7.2±0.3 (6.7-7.7) [n=15]	151 (123-268) [n=15]	707±76 (560-817) [n=19]	1.7 (1.2-40.3) [n=14]	26.2 (21.4-42.4) [n=14]
Carcinoma	42.1±6.5 (31.1-52) [n=32]	387 (213-794) [n=32]	12.4± 2.1 (9.4-18) [n=28]	7.0± 0.7 (6.1-8.8) [n=28]	276 (95-615) [n=28]	835±91 (652-982) [n=32]	3.2 (1.2-27.7) [n=21]	15.7 (11.2-26.1) [n=21}
Ref. Range	36-54	106-424	9-21	6-7.5	100-384	537-815	-----	-----
p-value	p=0.34	p<0.01	p=0.22	p=0.33	p<0.01	p<0.01	p=0.16	p<0.01

aPTT: activated Partial Thromboplastin Time; Fib: fibrinogen; HCT: Hematocrit; n: number of samples; PLT: platelet count; PT: Prothrombin Time; SD: Standard Deviation; TEG_{TG}: Thromboelastography parameter for Thrombus Generation. Asterisk denotes statistically significant difference.

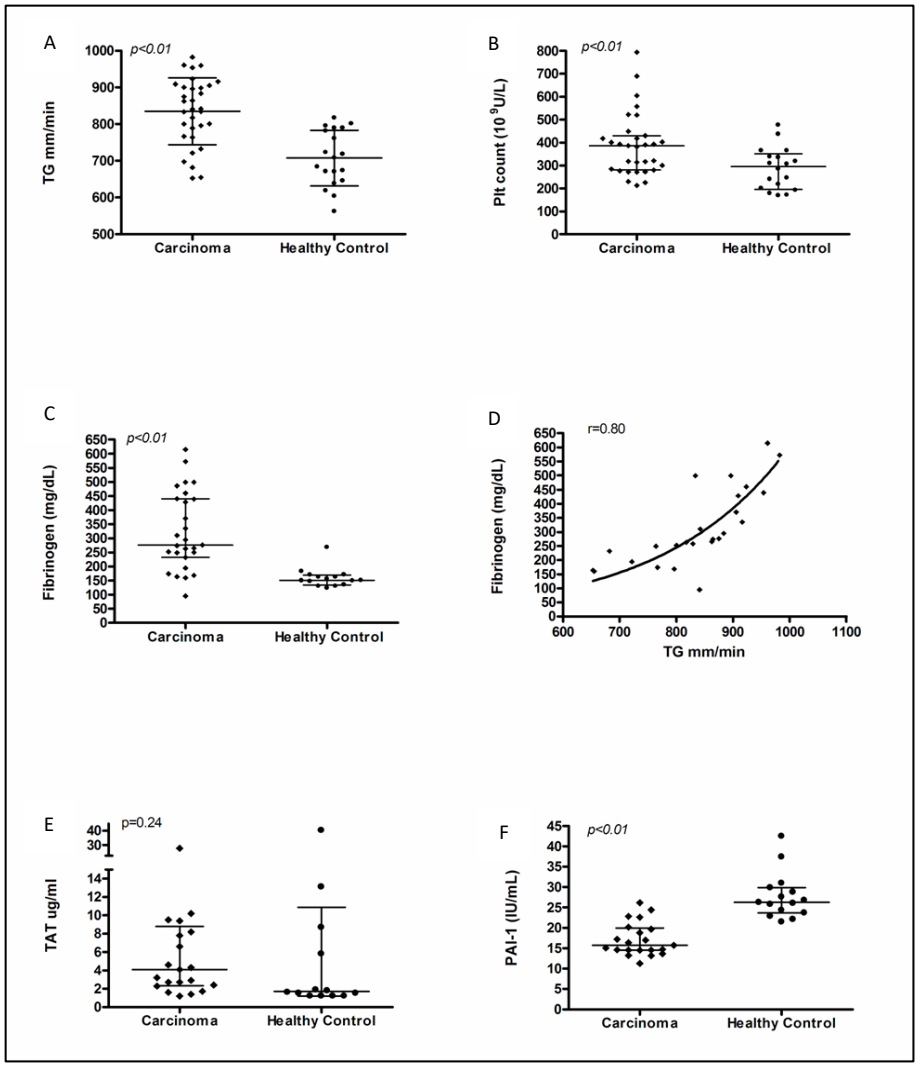

FIGURE 12. Vertical scatter plots showing the difference between the means for TEG_{TG} (Thromboelastography parameter for Thrombus Generation) (A), platelet count (B), fibrinogen (C), TAT: Thrombin anti-thrombin complexes (E), PAI-1: Plasminogen Activator-Inhibitor 1 (F) for carcinoma and healthy dogs and correlation curve of TG/fibrinogen (D). Horizontal bars indicate the Mean+/-SD for each group. *P-value* in italic denotes statistically significant difference between groups. Pearson's correlation factor TEG_{TG}/Fibrinogen is denoted with "r".

Table 9. Hemostatic parameters in dogs with metastatic carcinomas vs. dogs with non-metastatic carcinomas.

Results	HCT (%) Mean±SD (range) [n]	PLT x10^9/L Median (range) [n]	aPTT(s) Mean±SD (range) [n]	PT(s) Mean±SD (range) [n]	Fib mg/dl Median (range) [n]	TEG_{TG} mm/min Mean±SD (range) [n]	TAT µg/L Median (range) [n]	PAI-1 IU/ml Median (range) [n]
CA with met	42.4±1.6 (31-50) [n=17]	317 (240-605) [n=17]	12.7± 0.6 (10.4-18) [n=17]	7.0±0.6 (6.1-7.9) [n=17]	260 (160-615) [n=17]	837±23 (652-960) [n=17]	3.4 (1.2-8.2) [n=6]	15.7 (13.2-20.2) [n=6]
CA without met	43.4±1.7 (33-52) [n=15]	387 (213-794) [n=15]	12.3±0.5 (9.4-15.4) [n=15]	7.1±0.2 (6.1-8.8) [n=15]	274 (95-572) [n=15]	830±23 (682-982) [n=15]	7.8 (1.6-27.7) [n=9]	14.7 (13-22.8) [n=7]
R. Range	36-54	106-424	9-21	6-7.5	100-384	537-815	-----	-----
p-value	p=0.66	p=0.28	p=0.92	p=0.57	p<0.43	p<0.99	p=0.14	p=0.81

aPTT: activated Partial Thromboplastin Time; Fib: fibrinogen; CA: Carcinoma; HCT: Hematocrit; n: number of samples; Met: Metastasis; PAI-1:Plasminogen Activator-Inhibitor 1; PLT: platelet count; PT: Prothrombin Time; SD: Standard Deviation; TAT: Thrombin Anti-Thrombin complexes; TEG_{TG}: Thromboelastography parameter for Thrombus Generation.

4.4 THROMBOELASTOGRAPHY IN A DOG WITH HEMANGIOSARCOMA

We established a cytological (i.e., fine needle aspirate) and histopathological (i.e., excisional surgical biopsy) diagnosis of HSA in a 10-year-old male castrated Bichon Frise with multiple dark purple dermoepidermal nodules on the ventral abdomen and medial stifle areas, multiple small pulmonary nodules, and a solitary liver mass. The dog was treated with chemotherapy (Adriamycin-Cytoxan protocol). Forty-nine days after completion of four treatment cycles, the dog presented for recheck. CBC revealed anemia and mild thrombocytopenia. Chemistry profile showed no significant abnormalities. Analysis of hemostasis consisted of prolonged clotting times (i.e., PT, aPTT —out of range—) mild hypofibrinogenemia and

increased D-dimers (Table 10). A presumptive diagnosis of DIC was made based on these findings. TEG tracings correlated with the plasma-based test results showing hypocoagulability (i.e., prolonged PT and TEG_K —clot kinetics—; weaker clot with decreased fibrinogen levels, PLT count and lower TEG_{MA}–tracing amplitude–) and hyperfibrinolysis (i.e., increased D-dimers and TEG_{LY60} lysis parameters) (Table 11 and Figure 13). Based on these results, the dog was in the hyperfibrinolytic phase of DIC.

Table 10. Hemostasis panel in a dog with hemangiosarcoma.

Hemostasis profile	PT(s)	aPTT(s)	Fib (mg/dL)	PLT ($Ux10^9$/L)	Anti-thrombin (%)	D-dimers (ng/mL)
Case	11	out of range	85	92	89	1000 to 2000
Ref. Range	6.5 – 7.5	9 - 21	150 - 470	106 - 424	70 - 126	<250

Anti-thrombin, aPTT: activated Partial Thromboplastin Time, Fib: fibrinogen; D-dimers; PLT: platelet count and PT: Prothrombin Time.

Table 11. TEG results in a dog with hemangiosarcoma.

TEG	R (min)	K (min)	Angle (deg)	MA (mm)	G (dyn/cm^2)	LY30 (%)	LY60 (%)
Case	5.1	6.6	40.05	29.2	2.06×10^6	60.6	71.1
Ref. Range	1.0 – 6.1	0.9 –3.6	51.8 – 73.4	43.9– 67.9	$3.1\text{-}10.02 \times 10^6$	0 – 0.7	0 – 9.5

TEG (Thromboelastography) parameters: R: Reaction time; K: clot kinetics; Angle: angle of the TEG trace; MA: Maximum Amplitude; G value: shear modulus strength; LY30: percent lysis at 30 minutes after MA and LY60: percent lysis at 60 minutes after MA.

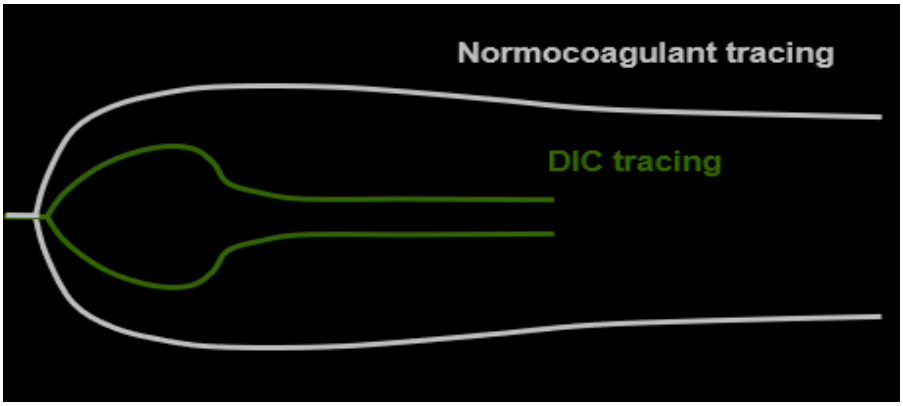

FIGURE 13. Prolonged clot kinetics, reduced amplitude and marked hyperfibrinolysis in the DIC tracing (green) compared to the normocoagulant tracing (white).

4.5 THROMBOELASTOGRAPHY AND DETECTION OF FIBRIN DEPOSITS IN THE MICROVASCULATURE OF HORSES

We evaluated 23 horses diagnosed with disorders frequently associated with coagulopathies using TEG performed less than 24 h before death. In addition, we included 5 horses considered healthy based on PE and with no history of coagulopathies aiming to power the correlation studies. Follow up necropsy was done no later than 24 h after death and samples from kidneys, lungs and liver were collected for histopathologic detection of fibrin deposits as described in the Material & Methods section. The most representative breed was Quarterhorse (n=14) followed by Standardbreed (n=2) and Hannoveranian (n=2). The male to female ratio was 1:1.6, including 9 geldings. Age ranged from 1 month to 24 years old with a median of 8 years. A detailed description of the clinical signs at presentation, macroscopic findings at necropsy and histopathologic findings (H&E) are shown below in Table 14.

PTAH stain revealed fibrin deposition in 4 out of 82 (4.8%) of the analyzed tissue sections. Positive detection was mostly observed surrounding the capillary of the pulmonary alveoli in 2 out of 4 (50%). The amount of fibrin deposition was considered mild in 3 out of 4 (score 1; n=3/4) positive sections and moderate in 1 out of 4 (score 2; n=1/4) (Figure 14).

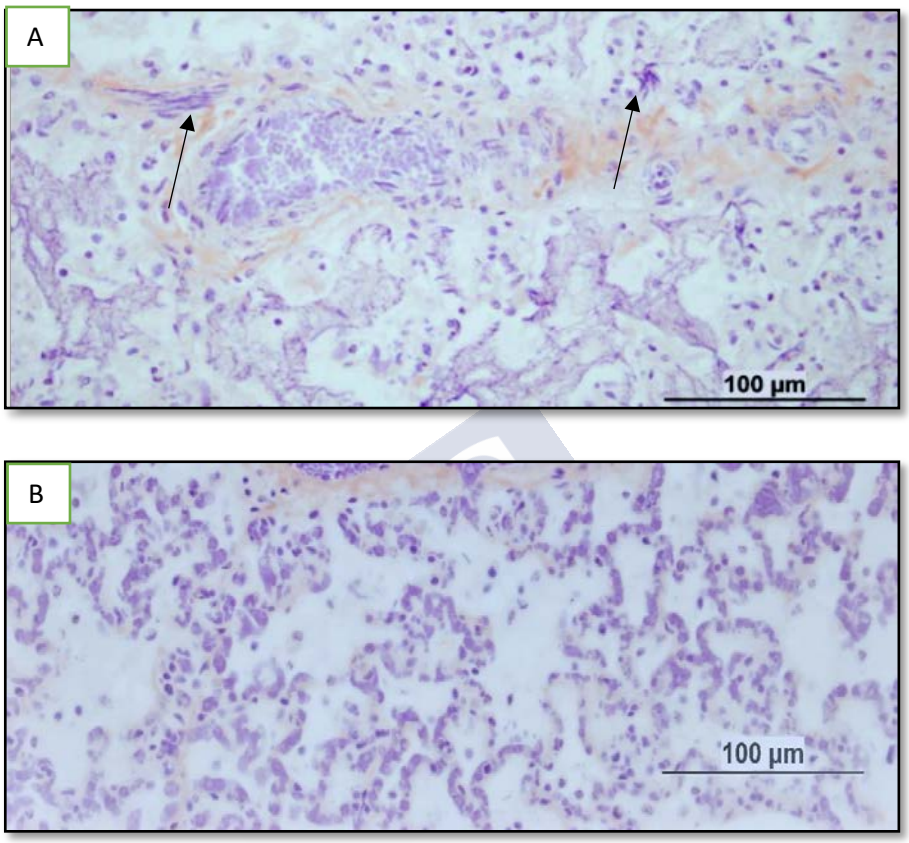

FIGURE 14. A) Mild fibrin PTAH staining (arrows) of the alveolar spaces in a section of lung containing pulmonary alveoli and blood vessels. B) Negative fibrin PTAH staining of the alveolar spaces in a section of lung containing pulmonary alveoli and blood vessels.

IHC allowed detection of fibrin deposits in 26 out of 82 (31.7%) of the analyzed tissue sections. Positive detection of fibrin deposits was mostly observed in the capillary of the pulmonary alveoli in 16 out of 26 (61.5%). The amount of fibrin deposition was considered mild in 15 out of 26 (score 1; n=15/27), moderate in 5 out of 26 (score 2; n=5/26) and marked in 6 out of 26 (score 3; n=6/26) (Figure 15). The reliability of either method to identify fibrin was found to be different ($p<0.001$)

on a McNemar exact test (x^2=506), and the Cohen's kappa coefficient was 0.06.

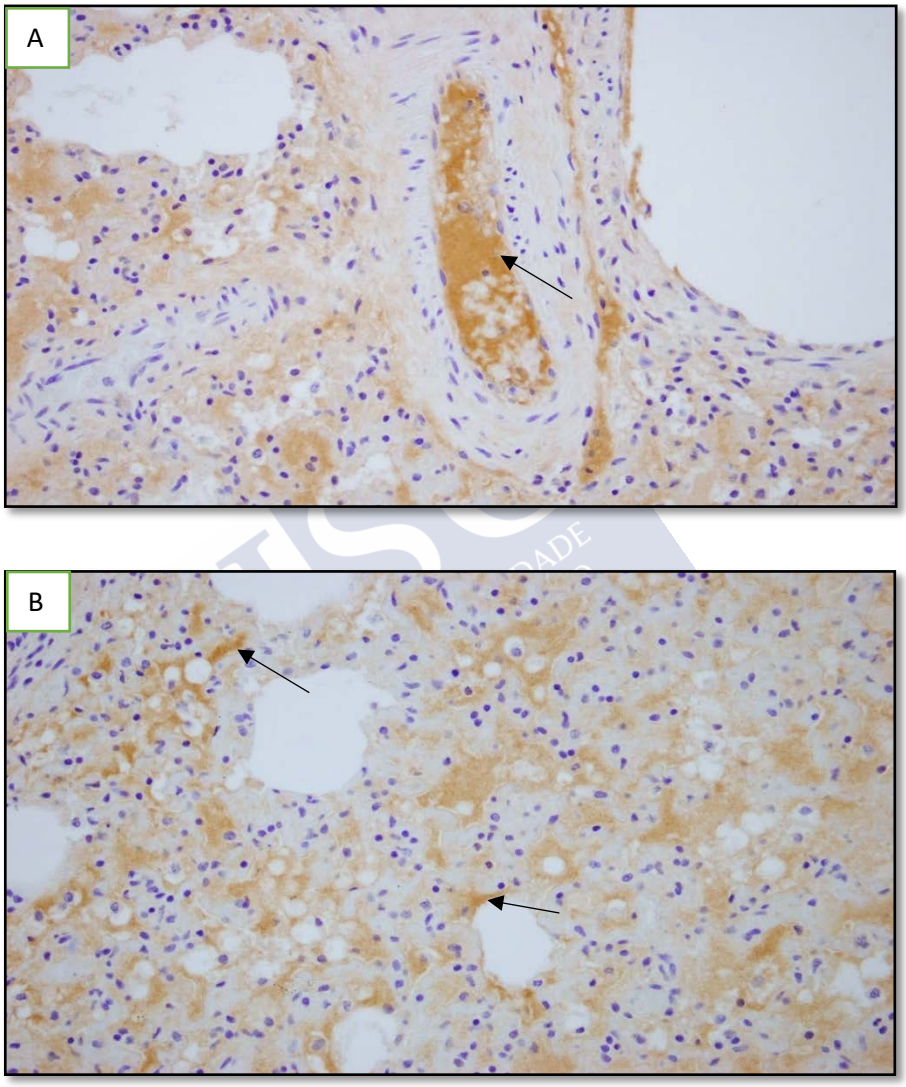

FIGURE 15. A) Marked fibrin IHC staining (arrow) of a clot formed in a pulmonary blood vessel (20x). B) Mild fibrin IHC staining (arrow) of the pulmonary alveolar spaces (20X).

Horses were further categorized in meta-scores from 0 to 4 based in the amount of fibrin deposition observed in the analyzed tissue sections following the guidelines shown in Table 2 of the Material & Methods section. Two out of 28 horses were not categorized because of missed tissue sections. One out of 26 horses (3.8%) was considered positive for fibrin microthrombi with the PTAH stain (meta-score 2; n=1/26), whereas 13 of 26 horses (50%) were categorized as positive with the IHC method (meta-score 1, n=2/13; meta-score 2, n=5/13; meta-score 3, n=6/13), as shown in Table 14. For horse categorization, the 2 methods agreed (positive and negative) 50% of the time with a Cohen's kappa coefficient of 0.06.

TEG evaluation of the study population showed that five horses were hypercoagulable (TEG_{MA} > 2SD + mean) and other five were hypocoagulable (TEG_{MA}< 2SD + mean). In regards of hemostasis panel, 12 horses were anemic and 5 had HCT values higher than normal range; 7 had thrombocytopenia and 6 thrombocytosis; 4 horses showed prolonged PT/aPTT; 9 had hypofibrinogenemia and 4 had hyperfibrinogenemia.

The correlation coefficients (r) between IHC, PTAH, TEG parameters, HCT, PT, aPTT, fibrinogen concentration and PLT count were not significant (Table 13).

Table 13. TEG and coagulation profile results including correlation coefficients in horses with disorders associated with coagulopathies.

	HCT (%)	PLT x10^9/L	PT(s)	aPTT (s)	Fib g/dL	R (min)	K (min)	Angle (deg)	MA (mm)	G dyn/cm^2	CL60 (%)	LY60 (%)
n	28	28	25	25	27	28	27	28	28	28	27	27
Min	8.00	8.00	9.90	26.1	60.0	4.10	1.50	3.1	2.5	130.3	-95.4	-1.20
25% Percentil	25.9	88.00	10.5	34.7	160.0	8.40	2.50	29.1	51.0	5209	80	0.0
Median	31.0	183.0	10.9	37.1	241.0	11.05	3.30	49.6	56.9	6618	90	2.90
75% Percentil	42.5	248.5	13.1	54.4	345.0	17.78	5.80	55.9	62.7	8429	99.3	7.60
Max	56.0	359.0	50.9	130	665.0	33.70	24.1	69.0	76.4	16157	100	12.7
r (IHC)	-0.12	-0.11	-0.30	-0.19	-0.03	-0.20	-0.16	0.1	-0.04	0.08	-0.3	0.38
r (PTAH)	-0.32	-0.31	-0.15	-0.34	0.20	0.32	0.33	-0.30	-0.30	-0.30	0.26	-0.24
Lab. Ref. values Mean ±SD	37.7±3.2	170±31.5	12±0.8	52.5±4.5	299.4±55.1	10.4±3.1	3.5±1.2	46.3±11	55.6±5.1	6429±1341	N/A	5.1±2.4

TEG (Thromboelastography) parameters: R, Reaction time; K, clot kinetics; Angle, angle of the TEG trace; MA, Maximum Amplitude; G value, shear modulus strength; CL60, clot lysis at 60 minutes; LY60, percent lysis at 60 minutes after MA. aPTT: activated Partial Thromboplastin Time; Fib: fibrinogen; HCT: hematocrit; n: number of samples; PLT: platelet count; PT: Prothrombin Time; r: correlation coefficient. Values shown as: minimal-Min, maximal-Max, median and 25% & 75% percentiles. Laboratory reference values shown as mean ± Standard Deviation (SD)

Table 14. Clinical signs at presentation and/or diagnosis, macroscopic findings at necropsy and histopathologic findings (H&E) in kidney, liver and lung samples. Phosphotungstic Acid Hematoxylin (PTAH) and Immunohistochemistry (IHC) meta-scores.

Case #	Clinical signs and/or Diagnosis	Necropsy Macroscopic Findings	Histopathology: Kidney	Histopathology: Lung	Histopathology: Liver	PTAH	IHC
1	Colic & severe epistaxis.	Hemoabdomen. Congestion & edema of multiple abdominal organs.	N/A	Autolysis, congestion & patchy alveolar edema.	Autolysis & cadaver bacilli.	0	0
2	Colic impaction/Right dorsal colonic displacement.	Pleural & pericardial ecchymoses & petechiae.	Mild interstitial lymphoplasmacytic inflammation & autolysis.	Congestion. Small number of clumped fibrin & neutrophils in pleural vessels. Mild interstitial hemosiderosis & occasional alveolar macrophages with hemosiderin.	Moderate periportal hemosiderosis, hemosiderin in Kupffer cells & some in hepatocytes. Minimal periportal bridging fibrosis & bile duct hyperplasia.	0	0
3	Diarrhea/Potomac Horse Fever.	Colitis.	Mild multifocal tubular mineralization.	N/A	Mild congestion.	N/A	N/A
4	Diarrhea/ Salmonellosis.	Adrenal cortical hemorrhages.	Focal cortical infarct with capsular fibrosis overlying.	Focal pleural abscess or pleuritis (orientation was	Locally extensive marked periportal to bridging fibrosis	0	2

		Hepatocellular necrosis. Renal infarct. Septicemia & thrombosis.	Membranous glomerulopathy & occasional glomerular sclerosis in area of infarct. Mild multifocal interstitial lymphoplasmacytic inflammation & fibrosis. Multifocal mineralization and several tubules contained neutrophils, protein casts. Multifocal segmental tubular necrosis and locally extensive medullary necrosis with surrounding hemorrhage (likely due to NSAID or steroid treatment).	difficult to assess). This region was characterized by marked necrotic cellular debris, degenerate inflammatory cells (likely neutrophils and foamy macrophages primarily but also lymphocytes and plasma cells), & fibrin that was surrounded by a thick band of fibrous connective tissue. The adjacent pulmonary parenchyma was characterized by bronchiolization, lymphoplasmacytic inflammation, clumps of fibrin in multiple vessels, occasional fibrin thrombi, alveolar macrophages & edema fluid.	& bile duct proliferation. Marked lobular collapse with loss of normal hepatic cord architecture. Multifocal hepatocellular necrosis (centrilobular to random). Moderate to marked lymphohistiocytic inflammation & marked reactive Kupffer cells. Centrilobular congestion. Occasionally possible fibrin in sinusoids.		

				Some bronchioles contain large numbers of degenerate neutrophils; neutrophils in interstitial vessels Peribronchiolar & peribronchial lymphocytes, plasma cells (BALT hyperplasia) & fibrosis.			
5	Lethargy/Hemorrhagic purpura & strangles.	Cerebro-cortical hemorrhages.	Multifocal mild tubular protein & mineralization in medullary tubules.	Marked autolysis. Locally extensive pulmonary infarct characterized by marked necrosis with multifocal mineralization of vessels & alveolar walls. Alveolar hemorrhage, fibrin, edema and multifocal foamy macrophages.	Marked autolysis & mild hemosiderosis.	2	0
6	Lethargy/ Equine Protozoal Myeloencephalitis.	NSF	Autolysis.	Mild autolysis.	Mild brown pigment in hepatocytes	0	0

					(probably hemosiderin).		
7	Lethargy & Painful muscular swellings/Clostridial myositis in hindquarters & Septicemia.	Severe fibrinosuppurative cellulitis. Necrotizing myositis. Multifocal subdural hemorrhages.	Autolysis & mild multifocal interstitial inflammation.	Autolysis & some acid hematin pigment.	Marked autolysis with small numbers cadaver bacilli. Acid hematin pigment.	0	0
8	Lethargy /Nephrosplenic entrapment. Toxic shock & colitis.	Severe necrotizing enteritis. Fibrin, thrombi and submucosal edema in the multiple sites along the intestine.	Autolysis.	Autolysis.	Autolysis with lots of hemosiderin & acid hematin.	0	0
9	Obtunded /Meningoencephalitis.	Intracranial hemorrhage. Fibrinosuppurative meningitis.	NSF	Congestion. Some fibrin in alveolar spaces & small number of macrophages.	Congestion.	0	3
10	Colic/Strangulating lipoma.	Strangulating lipoma of the colon.	NSF	Autolysis & mild peribronchiolar inflammation.	Mild hemosiderin.	0	0
11	Hind limb lameness/Vertebral fracture.	Subdural hemorrhage.	Mild membranous glomerulopathy.	Autolysis & alveolar edema.	Lots of acid hematin.	0	0

12	Colic.	Multifocal petechiation of the mesentery.	Mild membranous glomerulopathy.	Autolysis & alveolar edema.	Moderate to marked autolysis & cadaver bacilli.	0	0
13	Difficult breathing/Pneumonia.	N/A	NSF	Severe granulomatous to multifocally pyogranulomatous pneumonia (most consistent with Rhodococcus equi).	Moderate vacuolation of hepatocytes consistent with glycogen accumulation.	0	1
14	Lethargy & diarrhea/Colitis.	Fibronecrotic colitis. Infarctaction of kidneys. Pleural hemorrhagic necrosis.	Focal thrombus & cortical infarct. Locally extensive acute medullary necrosis & hemorrhage most consistent with NSAID or steroid treatment.	Moderate to marked multifocal alveolar edema & hemorrhage. Multifocal fibrin clumps in alveolar spaces with presence of macrophages, few neutrophils & degenerated cells. Neutrophils in alveolar walls. Occasional multifocal fibrinoid necrosis of vessel walls. Multifocal bacterial colonies suggestive of a response to endotoxemia.	Mild sinusoidal neutrophilia. Mild hemosiderin marked autolysis with cadaver bacilli. Acid hematin & mild sinusoidal neutrophilia.	0	3

15	Dysphagia and dysphonia/Laryngeal squamous cell carcinoma.	Hemorrhagic necrosis of a mass in the larynx.	NSF	Autolysis. Congestion & patchy alveolar edema.	NSF	0	2
16	Dyspnea & restless/ Fibrinosuppurative pleuropneumonia.	Fibrinosuppurative pleuropneumonia & pericarditis. Cranial mediastinal mass/abscess.	Moderate multifocal tubulointerstitial nephritis suggestive of an ascending bacterial infection. There is moderate multifocal interstitial lymphoplasmacytic inflammation with occasional small foci of tubular necrosis, degenerated neutrophils & necrotic cellular debris. Moderate tubular proteinosis. Mild to moderate membranoproliferative glomerulopathy.	Moderate to marked autolysis. Moderate patchy alveolar hemorrhage & edema. Lots of acid hematin.	N/A	0	0
17	Pale & abdominal distension/ Hemoabdomen & hepatic amyloidosis.	Liver amyloidosis.	Mild to moderate membranoproliferative glomerulopathy (unless this is a young horse).	Patchy edema.	Moderate to marked periportal to bridging fibrosis & bile duct proliferation with	0	0

			Mild interstitial lymphoplasmacytic inflammation.		pseudolobule formation. Multifocal coagulative necrosis & acid hematin.		
18	Colic/Intussusception & peritonitis.	Necrotic colon & peritonitis. Intestinal nematodiasis.	N/A	Moderate patchy edema & hemorrhage. Small number of megakaryocytes (can be seen with shock) & neutrophils in alveolar walls.	Lots of acid hematin. Mild to moderate sinusoidal neutrophilia. Locally extensive capsular fibrosis with neutrophils. Edema and fibrin consistent with peritonitis.	N/A	N/A
19	Loss of weight/Splenomegaly.	Abdominal hemorrhagic effusion & splenomegaly.	Mild membranous glomerulopathy. Mild multifocal interstitial lymphoplasmacytic inflammation.	Autolysis. Degenerate neutrophils in vessels & patchy edema.	Autolysis & occasional multifocal necrosis.	0	0
20	Ataxia/Compression C7-T1 (Wobbler Syndrome).	NSF	Mild membranous glomerulopathy.	Autolysis with cadaver bacilli & patchy edema.	NSF	0	3
21	Restless/Acute colic-cecocolic intussusception.	Diffuse colitis & intestinal tape worms.	Mild interstitial lymphoplasmacytic inflammation.	Patchy edema.	NSF	0	3

			Mild membranous glomerulopathy.				
22	Lameness/Right coxofemoral joint luxation.	Right coxofemoral luxation.	Mild interstitial lymphoplasmacytic inflammation. Mild membranous glomerulopathy Moderate membranoproliferative glomerulopathy (unless this is a young horse).	Autolysis. Hemorrhage & patchy edema.	NSF	0	2
23	Lameness/Chronic navicular disease.	NSF	Autolysis & mild multifocal interstitial inflammation.	Autolysis. Patchy congestion & edema. Mild chronic multifocal interstitial fibrosis. Thickening & hypercellularity of alveolar walls	Autolysis.	0	0
24	Healthy.	NSF	Mild interstitial inflammation.	Focal subpleural fibrosis. Mild patchy thickening & hypercellularity of alveolar walls.	Moderate periportal to bridging fibrosis & bile duct proliferation. Moderate hemosiderosis.	0	1
25	Healthy.	NSF	Mild membranous glomerulopathy. Minimal interstitial lymphoplasmacytic inflammation.	Mild thickening & hypercellularity of interstitium. Focal mild peribronchial lymphoplasmacytic inflammation.	Mild random multifocal lymphocytic inflammation. Mild hemosiderin in Kupffer cells.	0	3

				Lots of acid hematin.			
26	Healthy.	NSF	Mild membranous glomerulopathy. Minimal interstitial lymphoplasmacytic inflammation.	Autolysis.	NSF	0	2
27	Healthy.	NSF	Mild membranous glomerulopathy. Mild interstitial lymphoplasmacytic inflammation.	Mild multifocal lymphoplasmacytic peribronchial inflammation.	Mild hemosiderin in Kupffer cells. Lots of acid hematin.	0	2
28	Healthy with mild lameness.	Laminitis.	Occasional periglomerular fibrosis. Mild membranous glomerulopathy & rare glomerular sclerosis. Mild multifocal interstitial lymphoplasmacytic inflammation.	NSF	NSF	0	3

BALT: Bronchus-Associated Lymphoid Tissue; N/A: Not Available; NSAID: Non-Steroidal Anti-Inflammatory Drug; NSF: No significant findings

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5. DISCUSSION

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5. DISCUSSION

5.1 THROMBOELASTOGRAPHY IN RETIRED RACING GREYHOUNDS

It was recently shown that routine tests of hemostasis (i.e., aPTT, PT, PLT count) and platelet function (i.e., PFA) did not detect coagulopathies in RRG that suffered hemostatic complications after minor (i.e., gonadectomies) and major (i.e., amputation) surgical procedures.[84] We speculated that plasma based tests (i.e., aPTT, PT, fibrinogen) and individual cellular (i.e, PLT count and function) components used to routinely evaluate hemostasis may not be a reliable way to assess global hemostasis unless marked abnormalities are present. Thus, we hypothesized that a whole blood viscoelastic coagulation test (i.e., TEG) would be a more suited analyzer to identify coagulopathies and hemostatic differences in RRG.

Evaluation of hemostasis by TEG identified striking differences between RRG and non-RRG. The long TEG_K in RGG suggests lower enzymatic function and slower clot kinetics. The decreased TEG_{angle}, lower TEG_{MA}, and lower TEG_G typically are associated with thrombocytopenia or decreased interaction between fibrin and platelet assembly, and reduced clot strength (i.e., weaker clot). The diminished enzymatic clot function and kinetics observed potentially could explain the bleeding episodes in RRG.

Alternatively, the high whole blood viscosity in RRG (i.e., they have higher HCT: 41.81% in non-Greyhounds vs. 47.61% in RRG) could artefactually cause a decrease in the TEG_{MA} and TEG_G because of restriction of pin movement in the instrument. In addition, because RRG have less plasma per unit volume, the lower TEG_{MA} and TEG_G may be the result of excessive anticoagulant. However, according to the National Committee for Clinical Laboratory Standards Guidelines for

Collection, Transport, and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assay, anticoagulant concentration should be only adjusted in patients with HCT > 55%. [112] Only 2 RRG in this study had HCT > 55% (55.7 and 59%).

Although statistically significant differences in aPTT and PLT count were observed between the Greyhounds and non-Greyhounds in our study, those differences appear to be clinically irrelevant and are unlikely to have influenced the TEG tracing. For example, the mean difference for the aPTT between groups was approximately 1 second, and all results were within the reference ranges. Altered aPTT and PT results are considered clinically relevant only if they exceed by 25% those observed in the control samples. In this study, most control samples for aPTT were in the 11–12 second range; clinically relevant prolongation of the aPTT should have resulted in values of 14–16 seconds.[113]

5.2 THROMBOELASTOGRAPHY AFTER GONADECTOMY IN RETIRED RACING GREYHOUNDS

Once observed that RRG have different clotting TEG tracings if compared to non RRG, we decided to further analyze TEG tracings in RRG undergoing surgical procedures where severe bleeding episodes are relatively common with approximately 26% of RRG bleeding 24 to 48 h after gonadectomy. Doing so, we avoided any bias on our data analysis associated with breed differences.

We did not find significant differences between bleeders and non-bleeders pre surgery, but increases in TEG_{MA} and TEG_G observed in the non-bleeding RRG after surgery support the formation of a stronger clot in this group, an expected physiological hypercoagulable response to inflammation or stress induced by surgery.[88,90,114-116] Although the fibrinogen concentration was not a predictable parameter for bleeding in our study, TEG_G has been shown to have an excellent correlation (TEG_{Gf}, r^2=0.940) with the functional fibrinogen concentration.[117] Therefore, the total concentration of fibrinogen may not correlate accurately as well as the TEG_G value does with the amount of functional fibrinogen or with the fibrin networking (i.e., fibrin assembly). It is also

possible that postoperative changes to the HCT due to blood loss and intravenous fluid administration could have affected hemorheology and altered clot formation. Hemodilution after intravenous fluid administration of crystalloids may result in a hypercoagulable state on TEG; however, this effect is short lived and usually resolves soon after finishing the infusion. Based on these results, we postulated that the pathogenesis of this bleeding disorder in RRG occurs during the postoperative reactive phase of coagulation.

Clot formation depends on local cellular properties (e.g., endothelial cells, leucocytes) and availability of clotting factors, among others. The local thrombin concentration in the site of injury changes during clot formation; thus, defective thrombin generation patterns may result in abnormally structured clots that are associated with an increased risk of bleeding.[118] According to the results shown above and the cellular based theory of hemostasis, the authors of this manuscript proposed that if the bleeding syndrome in RRG is due to defective cell/protein interaction, the conventional quantitative and/or plasma-based coagulation test (i.e., aPTT, PT), PLT count and fibrinogen concentration, would not be predictive of the bleeding syndrome in RRG. In our study, the TEG was the only diagnostic test (i.e., TEG_{angle}, TEG_{MA}, TEG_G) that correlated with clinical signs of postoperative bleeding. Therefore, it may be a useful test to evaluate the perioperative risk of bleeding in RRG.

We should keep in mind as a limitation the low number of cases included in this research project, increasing the risk for a type II error on TEG parameters (i.e., TEG_K & TEG_R) and gender effect. A larger sample population will be necessary to determine the effect of some of these factors on bleeding in Greyhounds. An additional limitation of the present study is that the TEG tests were not performed in duplicate for logistical reasons (that is, only one TEG analyzer was available). However, ongoing studies at the authors' institution (data not published – internal data) revealed a coefficient of variation of less than 10% in duplicate samples for all the TEG parameters. Finally, to the author's knowledge, in the present study it was for first time documented a physiologic postoperative hypercoagulable reactive phase in dogs undergoing elective surgery using TEG.

5.3 THROMBOELASTOGRAPHY IN DOGS WITH CARCINOMA

Hypercoagulability in humans with malignancy has been related to the tumor's capacity to overexpress procoagulants, such as TF, via interaction of tumor cells with platelets, macrophages-monocytes, and vascular endothelial cells, thus resulting in a pro-thrombotic cell phenotype.[119-121] In veterinary medicine, the tests routinely used for evaluation of hemostasis, such as PT and aPTT, rarely detect hypercoagulable states.[96] Because of its ability to evaluate the cell-to-protein interaction during clot formation, TEG has been proposed as a useful tool to detect hypercoagulability in dogs.[86,102] Therefore, we further investigated TEG profiles in dogs with solid tumors to test the hypothesis of coagulation abnormalities in malignant tumor bearing dogs.

As expected, clotting times (i.e., PT, aPTT) were similar between carcinoma and non-carcinoma dogs, thus supporting the lack of ability for plasma-based hemostatic assays to detect hypercoagulability. We decided not to use activators of the coagulation cascade (e.g., synthetic phospholipids and silica, for aPTT; rabbit brain thromboplastin, for PT) in this study, since they may decrease the sensitivity of clotting times assays to detect hypercoagulability and override a mild activation due to the presence of activating or procoagulant substances. Regarding TEG profiles, TEG_{TG} was significantly higher in the dogs with carcinoma ($P<0.01$) ($TG_{mean\text{-}carcinoma} \pm SD$ vs. $TG_{mean\text{-}healthy} \pm SD$; 835 ± 91 vs. 708 ± 76). Eighteen dogs (56%) with carcinomas had TG values above the upper limit ($TG_{mean\text{-}healthy} + 2SD$) of the TG reference ranges for healthy age-matched dogs, confirming that TEG is a useful tool to detect hypercoagulability. In the present study, TEG_{TG} was used for the definition of hypercoagulability instead of TEG_{MA}, which is regarded as a valid parameter to detect hypercoagulability. TEG_{TG} derives from the TEG tracing, whereas TEG_{MA} is based on a single TEG tracing point. The inability to diagnose hypercoagulability in two hypercoagulable dogs with carcinoma based on TEG_{MA} may indicate a higher sensitivity of TEG_{TG} for the diagnosis of hypercoagulability.

Thrombocytosis was noticed in 46% of dogs with cancer in this study, a condition that has been also associated with the

hypercoagulable state of malignancy and with disorders commonly found in malignancy such as hypoferremia and chronic inflammatory disease.[122] The underlying mechanism of thrombocytosis and megakaryopoiesis in patients with malignancy is not completely understood, but it is suspected that release of pro-angiogenic factors (i.e., VEGF) and cytokines (i.e., IL-6, IL-1) may be involved.[123,124] It is also proposed that, under thrombin stimulation, platelets can contribute to the development of thrombocytosis by supporting the stimulation of bone marrow endothelial cells that release different cytokines, such as IL-6 and thrombopoietin that support megakarypoiesis.[125]

Hyperfibrinogenemia is common in dogs with malignancy, and it was the third most common hemostatic abnormality in our study affecting 32% of dogs with carcinoma.[126,127] In addition, the 61% of hypercoagulable dogs with carcinoma were hyperfibrinogenemic, therefore, highlighting the effect of functional fibrinogen in hypercoagulability and TEG_{TG}. It has been proposed that hyperfibrinogenemia is associated with the ongoing inflammatory response (e.g., high IL-6 concentration) in malignancy.[128] However, the correlation between fibrinogen concentration and tumor progression remains controversial and unclear.[129,130] Thrombin influences the fiber thickness and fibrin density of the clot; therefore, a higher thrombin concentration should result in a stronger fibrin structure, and the highly stable clots that are associated with thrombosis.[118]

TAT complexes are created after the production of thrombin and have been identified as a subclinical marker of thrombin formation.[131] To detect these complexes, we have used in this study a technique similar to those published for TAT activity in dogs.[132,133] Our results demonstrated no differences between the carcinoma and non-carcinoma dogs for TAT complexes, whereas TEG_{TG} was significantly higher in the carcinoma group. In contrast to our results, TEG studies in humans showed excellent correlation between TAT and TEG_{TG}.[134] The use of canine plasma in a human assay, and the amount of TAT complexes generated *in vitro* (i.e., possible inadequate sample incubation and/or mixing) may explain that poor correlation. That said, a recent publication indicated that TAT measurements with a similar

methodology to the one we used, were beneficial for detecting prothrombotic status in dogs with DIC.[135] Therefore, it is important to take into consideration that the number of samples included in the TAT analysis were lower than optimal and a type II error may be considered.

PAI-1 and other components of the fibrinolytic system play an important role as modulators of tumor angiogenesis and metastasis, but there is still controversy about the biological relevance of this protein in tumorigenesis.[136,137] High expression of PAI-1 is associated with poor prognosis in humans with a variety of cancers; however, McMahon et al. (2001) showed in an animal model study that lower concentrations of PAI-1 enhanced angiogenesis up to three-fold, but inhibited angiogenesis at high concentrations.[136,138,139] In our study, the PAI-1 activity in dogs with carcinoma was significantly lower than in the control group, a striking result that warrants future investigations to determine the role that this molecule plays on tumor progression.

5.4 THROMBOELASTOGRAPHY IN A DOG WITH HEMANGIOSARCOMA & DISSEMINATED INTRAVASCULAR COAGULATION

Not only thrombotic disorders have been associated with malignancy, but DIC has been reported as high as 62% in malignant solid tumors in humans, especially the chronic forms of DIC.[139] In dogs, DIC has been reported in up to 10-12% of those diagnosed with malignant solid tumors, and suffering from malignancy was found to be the leading cause for DIC.[140]

DIC has been described as occurring in two main stages. The initial "non-overt" stage is dominated by hypercoagulability, but the continuous consumption or biodegradation of clotting factors and platelets that takes place during this stage reaches a level where normal hemostatic integrity cannot be maintained, thus leading to a hypocoagulable or "overt" stage where clinical signs of spontaneous primary and secondary bleeding may be observed in the patient.[86,141,142] According to the International Society on Thrombosis and Hemostasis, the diagnosis of DIC may be established if an underlying disorder for the development of DIC is present and laboratory test shows reduced

PLT count, prolonged PT and aPTT, low fibrinogen and increased D-dimers.

We introduced a case report on a dog with HSA where TEG_K was markedly prolonged (over twice the upper reference value), and the PT and aPTT were also prolonged and beyond the upper limit of measurable range for aPTT; these findings are consistent with slow clotting kinetics associated with consumption of clotting factors or due to a low production of vitamin K-dependent coagulation factors in the liver. The TEG_{angle}, TEG_{MA}, TEG_G and PLT count were also below the reference range, reflecting the inability to form the necessary fibrin network to stabilize the clot. This is consistent with the reduced strength of the clot due to severe hypofibrinogenemia and mild thrombocytopenia from consumption and (though less likely for this case) for myelosuppression induced by the chemotherapeutic agents (i.e., doxorubicin, cyclophosphamide) to treat his HSA. The TEG_{LY30} and TEG_{LY60} were markedly increased, supporting hyperfibrinolysis. Therefore, TEG is a valuable diagnostic test for DIC in dogs with an underlying disease that would predispose the dogs to develop a coagulopathy, and/or for classifying DIC (i.e., overt vs. non-overt).[84]

In a recent study, hypercoagulability defined as a TEG_G > 7.2k dyn/cm^2 was the most common finding on TEG in dogs with suspected DIC characterized by a prolongation of aPTT and/or PT, low fibrinogen or increased D-dimers.[84] The authors of that study, categorized the overall hemostatic state based solely on a single TEG parameter (i.e., G value). This is a legitimate method for the categorization of DIC, but the criteria for TEG analysis of dogs with DIC could include the entire TEG tracing, or an index/parameter that represents the entire evaluation of the TEG tracing (i.e., area under the curve-AUC).

Fibrinolytic parameters (i.e., TEG_{LY30}, TEG_{LY60}) may add important information for treatment and prognosis. For example, the use of antifibrinolytics is controversial in veterinary medicine, but it is recommended and has a standardized protocol for human patients with DIC characterized by a primary hyperfibrinolytic state and bleeding.[143] In addition, diagnosis of different lytic patterns for coagulative disorders has been shown to have prognostic value using similar

techniques such as thromboelastometry.[144] Both methods, thromboelastometry and TEG, were considered gold standard techniques for hyperfibrinolysis detection in human medicine.[145,146] A recent case report in humans demonstrates how TEG was used to diagnose a hyperfibrinolytic phase of DIC, monitor and treat with antifibrinolytic therapy (i.e., Aminocaproic Acid), blood products (i.e., cryoprecipitate) and others (i.e., Factor VIIa) with success.[147] Newer publications confirmed the diagnostic value of TEG to detect DIC in patients with malaria and for the study of DIC following radiation exposure in a porcine model.[148,149] DIC classification and diagnosis using TEG may be extremely useful to establish an accurate treatment (e.g., anticoagulants, plasma, antifibrinolytics), monitoring and prognosis in veterinary medicine.

5.5 TEG AND FIBRIN DEPOSITS IN THE MICROVASCULATURE OF HORSES

It has been suggested in several studies that fibrin deposits accumulated in the microvasculature of organs (e.g., kidney, liver, lung) are associated to the hypercoagulable state observed in DIC.[150-152] In our study, however, no significant correlation between the procoagulant state (determined by TEG) and the fibrin deposits (detected by IHC and PTAH) was found. This could be explained because of a low number of horses in hypercoagulable state, only 5 based on TEG. Similarly, Dunkel et al. (2010) encountered difficulties to observe hypercoagulable TEG profiles in horses presumed to be in procoagulant state.[153] It is also possible, but less likely, that we sampled none hypercoagulable horses in DIC state with marked fibrinolysis, reduced amount of fibrin deposits and no signs of bleeding. Further studies are warranted to detect if 24 h time period is adequate to standardize correlation of coagulation changes to pre- and post-mortem findings (TEG and necropsy), and if TEG is a useful tool to diagnose hypercoagulability in horses as it is for dogs. In addition, those future studies should consider including a larger number of horses with suspected and proven coagulopathies, as it became obvious from our data that we underpower our statistical analysis, regardless of the limitations of current coagulation analyzers to detect coagulopathies.

Our results showed minimal detection of fibrin deposits in the sections using PTAH staining, compared to the results obtained with IHC. For example, some of the microscopic descriptions of the hematoxylin and eosin stained tissue sections identified fibrin deposits in the vessels, as well as fibrin thrombi, which were also detected by IHC, but not by PTAH. Even though earlier publications proved the efficiency of the PTAH technique to detect fibrin deposits on sections,[152] there are many variables (i.e., albumin, lipoproteins) that may result in a lower accuracy and specificity of the staining.[154-156] Therefore, our observations point to a more precise detection of fibrin deposits by IHC compared to PTAH staining. This low correlation in terms of detection of fibrin deposits between one stain and another was reflected in the McNemar exact test (x^2=506; $p<0.01$), and the Cohen's kappa coefficient was 0.06. PTAH staining revealed fibrin deposits in 4 of 82 (4.8%) of the tissue sections analyzed. Positive detection was observed mainly around the capillaries of the pulmonary alveoli. In the case of IHC, it allowed the detection of fibrin deposits in 26 of 82 (31.7%) of the analyzed tissue sections. Positive detection of fibrin deposits was also observed mainly in association with the capillaries of the pulmonary alveoli in 16 of 26 (61.5%) sections analyzed. As shown by Cotovio et al. (2007), the higher deposition of fibrin in the lung may be related to some extent to the particular feature of the fibrin deposition throughout the capillary and medium-size blood vessels of the lungs, possibly related to the percentage of cardiac output passing through the organ or with the alterations in the distribution of blood flow, as occurs during shock (shock organ in horses).[110,152]

Another interesting finding from our study was that 8 of the 23 horses had a gastrointestinal disorder (e.g., colic syndrome, salmonellosis), but no correlation was found between the detection of fibrin deposits and the type of pathology. Furthermore, fibrin deposits detected by IHC were frequently found in those horses considered healthy because they did not suffer from a known pathology. These results contradict those published in a previous study in which they found a greater amount of fibrin deposits in tissues of horses with gastrointestinal disorders with a poor prognosis, while fibrin deposits were not observed in horses with colic of better prognosis.[110]

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6. CONCLUSIONS

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The comprehensive analysis of the results obtained in the multiple studies shown above, allow us to announce the following conclusions:

1. In RRG, clotting kinetics is slower and clot strength is weaker than in non-RRG, supporting the increased tendency to bleed observed after minor trauma or surgical procedures in this breed.
2. A physiologic postoperative phase with trend towards hypercoagulability is observed in non-bleeder RRG after gonadectomy.
3. TEG, by virtue of its parameters TEG_{angle}, TEG_{MA}, and TEG_{G}, is a reliable test to evaluate the perioperative risk for bleeding in RRG.
4. Hypercoagulability, thrombocytosis and hyperfibrinogenemia are common hemostatic abnormalities in dogs with carcinoma.
5. Hyperfibrinogenemia characterizes the hypercoagulable state in carcinoma dogs as measured by TEG_{TG}, a valid parameter to diagnose hypercoagulability.
6. TEG is a bedside diagnostic tool useful to classify the hypocoagulable stage with hyperfibrinolytic phase of “overt” DIC in dogs.
7. There is no correlation between TEG and the amount of fibrin deposits detected by PTAH and IHC in the vasculature of horses. Thus, in this study, no prognostic value could be validated for TEG in horses at risk for coagulopathies.

8. The concordance of the histological techniques PTAH and IHC in identifying fibrin deposits in horses is surprisingly poor. According to our methodology and results, we favour the use of IHC for the detection of fibrin deposits on tissue sections rather than the use of PTAH staining.

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