Evaluation of hemostasis in hyperthyroid cats

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ABSTRACT

Background: Hyperthyroid cats are predisposed to thrombus formation. The mechanism for thrombogenesis is currently unknown, but could be associated with altered hemostasis as seen in hyperthyroid humans.
Objective: The purpose of this study was to evaluate markers of hemostasis in hyperthyroid cats compared to healthy cats, and in hyperthyroid cats before and after treatments with radioactive iodine (RIT).
Methods: Twenty-five cats with hyperthyroidism and 13 healthy euthyroid cats > 8 years of age were studied. Prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, antithrombin (AT), D-dimers, thrombin-antithrombin complexes (TAT), von Willebrand Factor antigen (vWF:Ag), and activity of factors VIII and IX were measured. An echocardiogram was performed in all cats and healthy cats with abnormal echocardiograms were excluded. Measurements of hemostasis were evaluated again in 7 cats $\geq$ 6 months after RIT and deemed to have restored euthyroid status.
Results: There is a significant likelihood of being in hypercoagulable state based on hyperthyroid state ($P = 0.019$) and serum T4 level is significantly associated with predicating hypercoagulability ($P = 0.043$). Hyperthyroidism is associated with significantly higher median fibrinogen concentration ($P < 0.0001$), higher median AT activity ($P < 0.0001$), and higher median vWF:Ag level ($P = 0.01$) with all values decreasing significantly post-RIT. Fibrinogen and AT had a strong positive correlation with serum T4 value ($r = 0.79$; 95% CI 0.63 - 0.89 and $r = 0.70$; 95% CI 0.50 - 0.84, respectively). Presence of an abnormal echocardiogram in hyperthyroid cats was associated with a significantly higher median fibrinogen concentration ($P = 0.03$). Echocardiographic status did not have a significant impact on the remaining hemostatic markers in hyperthyroid cats.
Conclusions: These results provide evidence of altered hemostasis and hypercoagulability in hyperthyroid cats that do not appear to be solely attributed to cardiac abnormalities. These differences of altered hemostasis resolved after radioiodine therapy, but further studies are warranted to determine if hypercoagulable state resolves.

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GENERAL ABSTRACT

In feline hyperthyroidism, there is a predisposition for thrombus formation. An alteration of hemostasis has been documented in hyperthyroid humans, but despite reports of thrombus formation in hyperthyroid cats, the underlying mechanism is currently unknown. Hyperthyroidism can lead to cardiac abnormalities that could possibly contribute thrombus formation, although thrombus formation has occurred in hyperthyroid cats without detected abnormalities.

The goal of this study was to evaluate markers of hemostasis in hyperthyroid cats presenting for radioiodine therapy to evaluate for presence of hypercoagulability. Twenty-five hyperthyroid cats were evaluated with hemostasis panels and echocardiograms. The results were compared to a group of 13 healthy cats. Markers of hemostasis and echocardiograms in 7 hyperthyroid cats were also compared to results 6 months or greater post-radioiodine therapy.

There was evidence of altered hemostasis and hypercoagulability in hyperthyroid cats. The alterations noted resolved after radioiodine therapy and do not appear to be solely attributed to cardiac abnormalities seen in hyperthyroid cats.

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ABBREVIATIONS

2D	two-dimensional
GPIa/IIa	glycoprotein Ia/IIa
GPIIb/IIIa	glycoprotein IIb/IIIa
ANOVA	one-way analysis of variance
APCR	activated protein C resistance
aPTT	activated partial thromboplastin time
AT	antithrombin
ATE	arterial thromboembolism
GPIa/IIa	glycoprotein Ia/IIa
GPIbα	platelet glycoprotein Ibα
GPIIb/IIIa	glycoprotein IIb/IIIa
GPVI	glycoprotein VI
HCM	hypertrophic cardiomyopathy
HMWK	high-molecular-weight kininogen
LA:Ao	left atrium to aorta ratio
NET	neutrophil extracellular trap
PAI-1	plasminogen activator inhibitor
PAR	protease activated receptor
PK	prekallikrein
PT	prothrombin time

RI	reference interval
RIT	radioactive iodine
ROS	reactive oxygen species
ROTEM	rotational thromboelastometry
T3	3,5,3’-triiodothyronine
T4	thyroxine
TAFI	thrombin activatable fibrinolysis inhibitor
TAT	thrombin-antithrombin complexes
TE	thromboembolism
TEG	thromboelastography
TFPI	tissue factor pathway inhibitor
THRB	thyroid hormone receptor β
tPA	tissue plasminogen activator
VMCVM	Virginia-Maryland College of Veterinary Medicine
VTE	venous thromboembolism
vWF	von Willebrand factor

CHAPTER 1: INTRODUCTION OF HYPERTHYROIDISM AND COAGULATION

In humans, hyperthyroidism affects coagulation and the heart. Hyperthyroid cats are predisposed to thrombus formation. The mechanism for thrombogenesis in hyperthyroid cats is currently unknown, but could be associated with altered hemostasis as seen in hyperthyroid humans. Currently, there are few studies pertaining to coagulation alterations in hyperthyroid cats as well as the potential consequences. The purpose of this study was to evaluate markers of hemostasis in hyperthyroid cats compared to healthy cats, and in hyperthyroid cats before and after treatments with radioactive iodine (RIT).

CHAPTER 2: LITERATURE REVIEW

A. Physiology of Hemostasis

a. Primary hemostasis

Primary hemostasis involves the promotion of rapid formation of platelet plugs and clots through interaction with the endothelium. Platelets will adhere to sites of vascular injury where there is exposure of the subendothelial extracellular matrix. The subendothelial matrix contains adhesive macromolecules that act as ligands for different platelet surface receptors. The subendothelial substrates that bind platelets include von Willebrand factor (vWF), thrombospondin, laminin, collagen, and fibronectin.[1–6] To adhere to the extracellular matrix, platelets undergo the processes of tethering, rolling, activation, and firm adhesion.[7] Rolling adhesion is the first step in recruiting platelets to the site of injury. For platelet tethering, interactions between adhesion molecules must form rapidly and for rolling, the interactions must rapidly break to provide a checkpoint before committing to firm adhesion. Cell adhesion occurs by reversible interactions between ligands and platelet surface receptors in extracellular

matrix. These events occur by interaction of platelet glycoproteins and integrins with subendothelial proteins. Binding of platelet glycoprotein Ibα (GPIbα) to vWF recruits fast flowing platelets in high shear blood flow at sites of vascular damage.[8] Collagen is an important component in initiation of platelet adhesion and aggregation with glycoprotein VI (GPVI) and glycoprotein Ia/IIa (GPIa/IIa) playing a role in platelet-collagen interactions.[9] Glycoprotein VI is important for stable platelet adhesion on exposed extracellular collagen due to its induction of strong signaling via the Fc receptor γ-chains which are present as a complex with GPVI on the platelet surface.[10,11] Upon binding to collagen, GPIa/IIa results in platelet activation by enhancing and reinforcing GPVI-collagen interactions and collagen binding to GPIa/IIa will trigger signaling that activates glycoprotein IIb/IIIa (GPIIb/IIIa).[12,13] These actions reinforce GPIa/IIa and GPVI's activity leading to further adhesion and activation on collagen.[14] Lastly in platelet adhesion, there is firm arrest on the extracellular matrix that shifts several β1 and β3 integrins to a ligand binding state that is high affinity. The most abundant integrin is GPIIb/IIIa on platelets, which along with platelet aggregation, also mediates firm adhesion of platelets after binding to vWF.[15]

The GPIb-IX-V complex is found on the surface of platelets is composed of the subunits GPIbα, GPIbβ, GPIX and GPV that binds a number of ligands in addition to vWF. The GPIb-IX-V complex contributes to platelet activation with binding of the ligand thrombin, a serine protease.[16] This mechanism of activation is currently not completely understood. Thrombin has been shown to also activate platelets through the binding to protease activated receptors (PARs). In humans, platelets express PAR1 and PAR4 that trigger platelet activation and aggregation with when they are activated.[17,18] The main receptor for thrombin on human

platelets is PAR1 on human platelets and at high thrombin concentrations, PAR4 contributes signaling and may maintain signaling and a prolonged effect in platelets.[19]

Platelet aggregation occurs after platelet adhesion and activation with the presentation of a procoagulant surface that promotes forming a hemostatic plug that is fibrin-rich. Stable binding of platelets will trigger pathways of activation involving signal transduction and tyrosine kinases through G-coupled receptors.[20–22] This leads to increased cytosolic calcium levels, rearrangements of the cytoskeleton, and integrin activation, which are all critical for platelet activation. Shape change, aggregation, and secretion of granules all occur with increases in intraplatelet calcium levels.

A release reaction takes place after platelet binding and signaling to the platelet cytoplasm with platelet granules fusing to the outer membrane and releasing their contents to the regional environment resulting in amplification of the platelet activation process.[23] The granules identified in platelets are α-granules, dense (δ) granules, and lysosomes (λ-granules). Alpha-granules contain growth factors chemokines, anti- and pro-thrombotic molecules, and anti- and pro-angiogenic molecules.[24] Alpha-granules store high molecular weight multimers of vWF and fibrinogen, which increase cross-linking of platelets via GPIb/IX/V and GPllb/IIIa, respectively.[25] The α-granules also contribute to secondary hemostasis with the release of high molecular weight kininogens, prothrombin, and factors V, XI, XIII.[23] Dense granules contain fewer proteins and contain calcium ions, histamine, and serotonin.[26] Dense granules secrete adenosine diphosphate which is a major platelet activator that binds to the $P2Y_1$ and $P2Y_{12}$ receptor causing platelet aggregation and shape change through G_q mediated phospholipase C-β2 activation.[27] The platelet $P2Y_{12}$ receptor for adenosine diphosphate is important for platelet

function, hemostasis, and thrombosis and drugs inhibiting $P2Y_{12}$ receptors are potent antithrombotic drugs.[28] Lysosomes hold enzymes including serine peptidases, carbohydrases, and phosphatases.[26]

Platelet aggregation follows platelet adhesion and activation which leads to the formation of a fibrinogen-rich thrombus. Platelet aggregation involves many ligands (fibrinogen, fibronectin, vWF), receptors (GPIbα and GPIIb/IIIa) and platelets in different activation states.[29] Three distinct mechanisms have been identified that initiate platelet aggregation on adherent platelets depending on the shear rate.[30] Platelet aggregation is mediated by GPIIb/IIIa fibrinogen interaction at low shear rates (<1000 s^{-1}).[29,31] The shear rates between 1000 and 10,000 s^{-1}, a beginning phase depends on the adhesive function of GPIbα and GPIIb/IIIa and is mediated by reversible platelet aggregate formation. The next step at this sheer rate depends on platelet agonists generation and the irreversible activation of GPIIb/IIIa.[29,31] At high shear rates (>10,000 s^{-1}), platelet aggregation is mediated by vWF- GPIbα interactions and can occur without the presence of GPIIb/IIIa.[29,31] Depending on the hemodynamic conditions, platelets will be exposed to a range of low flow situations with low shear rate in venules and large veins to moderate shear rate in small arterioles to high flow with high shear rate in stenosed arteries.

b. Secondary hemostasis

Coagulation proteins consist of a serine protease with different protein domains. Protein C, protein S, protein Z and factors II, VII, IX, and X have vitamin K-dependent glutamic acid domains on the amino terminus of the protein.[32] The domains on the amino terminus of the protein modify to allow calcium to bind to these proteins in order to bind to phospholipid

surfaces. A quaternary complex is formed beginning with the enzyme binding to a cofactor bonded by calcium to the phospholipid surface which improves efficiency of the reaction.[33]

Activation of secondary hemostasis occurs at the same time with platelet activation at vascular injury sites.[34] Secondary hemostasis involves formation of fibrin by the coagulation proteins on the surface of activated and aggregated primary platelet plugs.[34] A series of enzymatic steps comprises the coagulation cascade and more recent research has revealed fibrin formation in vivo differing from test-tube models of coagulation and classic literature.[34]

The pathways described in older literature are compose of the extrinsic, intrinsic, and common pathways.[34] The extrinsic pathway consists of factor III (tissue factor) binding to factor VII, and this tissue factor-factor VII complex then activates factor X. The intrinsic pathway is dependent on different enzymes that results in factor XII activating factors XI, IX, and VIII. Then, both pathways activate factor X, initiating the common pathway. In the common pathway, activated factor X complexes with factor V to activate factor II (thrombin) to cleave factor I (fibrinogen) into activated factor I (fibrin).[34] Contradictory findings in clinical observations led to development of an alternative pathway.[35,36]

In the cell-based model of coagulation, the 3 phases recognized are initiation, amplification, and propagation. Tissue factor is required to initiate secondary hemostasis and is in the extracellular matrix between and beneath endothelial cells and is exposed after endothelial injury.[37,38] Tissue factor will bind to factor VII which speeds up the activation of factors IX and X.[38] Factor X is activated by the factor VIIa-tissue factor complex in areas with high tissue factor concentrations, but at low concentrations with minor vascular injuries, factor IXa-/factor VIIIa-dependent activation is more prominent.[35,36] Activated factor X activates prothrombin on a

phospholipid surfaces with activated facto V, its cofactor, that is released from activated platelets' α-granules.[39]

During amplification, the next phase of the cell-based model, once thrombin has been generated, thrombin is available for activation of platelets. The platelet activation results in shape change, creation of procoagulant membrane surface, and release of granule contents. On the surface of platelets, the propagation phase then occurs with aggregation of platelets occurring by ligand expression allowing this interaction. Activated factor IX can bind to factor VIIIa on the platelet surface. This complex begins to generate activated factor X, which rapidly binds to activated factor V that cleaves prothrombin to thrombin.[40]

Thrombin converts fibrinogen into fibrin. Positive feedback is provided by thrombin with activating thrombin activatable fibrinolysis inhibitor (TAFI) and factors V, VIII, XI, and XIII.[41] Negative feedback is also provided by thrombin in activating protein C and promoting fibrinolysis.[42] Thrombin converts fibrinogen into fibrin monomers by clipping off fibrinopeptides A and B.[43] This exposes polymerization sites in order to bind other fibrin monomers to form fibrin polymers.[43] Factor XIII will then stabilize the clot by forming glutamyl-lysine bridges between fibrin polymers.[44]

c. Fibrinolysis

Breaking down blood clots to prevent thrombi from becoming too large and to prevent thrombosis in undesirable anatomical sites is the responsibility of the fibrinolytic system.[33] Fibrinolysis is a balance of activators, inhibitors, inactivators, inactive proenzymes, and active enzymes.[45] Fibrinolysis is initiated almost concurrently with coagulation and is responsible for patency of the vasculature by maintaining blood in a fluid state.

The main proteins involved in the fibrinolytic system are plasminogen activator inhibitor (PAI-1), plasmin, urokinase, tissue plasminogen activator (tPA), and alpha$_2$ antiplasmin.[45] Produced by the liver, plasmin is a serine protease that cleaves bonds in fibrinogen and fibrin.[46] The inactive precursor, plasminogen, circulates until it is converted to plasmin by urokinase or tissue plasminogen activator.[46] Plasmin is formed after plasminogen is activated when it is cleaved into heavy (A) and light (B) chains.[47] Endothelial cells produce tissue plasminogen activator.[46] PAI-1 is made by the liver and endothelial cells and its activity is to bind and inactivate tPA.[48] Alpha$_2$ antiplasmin is also made by the liver and its activity is to bind and inactive plasmin.[49]

Within developing clots, activation of fibrinolysis occurs where plasminogen and plasminogen activators bind to fibrin.[47] In the tPA conversion of plasminogen to plasmin, it is greater when both are bound to the fibrin clot.[50] Additional bindings sites are uncovered after initial proteolysis of fibrin, which enhances further binding of plasminogen and its activators.[46] Plasmin is protected from the action of alpha$_2$-antiplasmin when plasmin is bound to fibrin.[51] A thrombus that forms will incorporate plasminogen into the clot.[46] Endothelial cells nearby release tPA into the clot and tPA binds to fibrin leading to conversion of plasminogen to plasmin.[52] Plasmin is capable of hydrolyzing fibrinogen and fibrin with the resulting formation of fibrin degradation products or fragments.[53] Fibrinolysis is confined to the thrombus itself by excess tPA and excess plasmin escaping into plasma being inactivated by PAI-1 and alpha$_2$-antiplasmin, respectively.[54]

d. Anticoagulation and antifibrinolysis

Anticoagulant and antifibrinolytic mechanisms prevent fulminant thrombotic or hemorrhagic outcomes. These mechanisms regulate the activation of zymogens to proteases, release of enzymes into the blood, amplification of enzyme activation, and neutralization of activated enzymes with inhibitors. The proteins that inhibit thrombosis at different steps of the coagulation cascade are protein C, protein S, tissue factor pathway inhibitor (TFPI), and antithrombin (AT).[33]

TFPI is a Kunitz-type, multivalent, serine protease inhibitor that inhibits early phases of the procoagulant response.[55] Tissue factor pathway inhibitor complexes with factor Xa that subsequently forms a complex with tissue factor VIIa resulting in activation of the tissue factor-VIIa complex.[56,57] The coagulation cascade is suspected to continue via thrombin activating factor XI that activates factor IX leading to more thrombin generation.[35] Therefore, the coagulation cascade is incompletely inhibited via TFPI.

AT binds and inactivates the serine proteases in the coagulation cascade. Adding natural heparan sulfate or exogenous heparin, there is an increase in its function by dramatically augmenting AT's binding and neutralization of serine proteases.[58] AT is an inhibitor of thrombin, kallikrein, plasmin, and factors Xa, IXa, XIa, and XIIa with the greatest activities against thrombin and factor Xa.[59] Because it is a major naturally occurring inhibitor of coagulation, deficiencies result in excessive thrombin generation. When thrombin is generated, AT can bind thrombin to form thrombin-antithrombin complexes (TAT).[59]

The components of the protein C anticoagulant pathway include protein C, protein S thrombin, thrombomodulin, and endothelial cell protein C receptor. Protein C is a serine protease that irreversibly inactivates factors Va and VIIIa. As an essential cofactor of protein C,

protein S regulates protein C's activity.[60,61] Unlike most other anticoagulant factors, protein S is not a serine protease. Protein S circulates as both an unbound form bound to the C4B-binding protein.[62] The free form only will serve as protein C's cofactor. The protein S amount that exists in the free form is about 40% and alterations in this percentage has been noted to be responsible for many hypercoagulable states.[63] Thrombomodulin binds thrombin, which directly inhibits its cell activation and clotting while augmenting protein C and TAFI activation.[64] Protein C receptor on the endothelial cell increases protein C activation by presenting it to the thrombin-thrombomodulin activation complex.[64] Once activated protein C dissociates from the endothelial receptor, it will bind to protein S where it inactivates factors Va and VIIIa.[64]

B. Hypercoagulability

Virchow's triad consists of three predisposing factors that may lead to thrombosis, which include hypercoagulability, blood stasis, and endothelial injury. Hypercoagulability is defined as an individual having a heightened potential to develop arterial or venous thrombosis due to abnormalities in the coagulation system. The term thrombophilia is an acquired clinical phenotype presenting in individuals as an increased risk in developing recurrent thrombosis.[65] Hypercoagulability is a laboratory phenotype with activation of platelets, endothelial cells, and fibrinolysis identified in vitro. Hypercoagulability can be provoked by blood stasis, inflammation, drugs, sepsis, surgery, and atherosclerosis and occurs in inherited and acquired thrombophilia.[66] Inherited causes in humans include Factor V Leiden, protein C and S deficiency, AT deficiency, prothrombin gene 20210G/A mutation, elevated factor VIII, sticky platelet syndrome, and hyperhomocysteinemia.[67–74] Acquired causes include pregnancy,

malignancy. lupus anticoagulants/antiphospholipid antibody syndrome, trauma, oral contraceptive and hormone replacement therapy, infectious and inflammatory disease, heparin-induced thrombocytopenia, prolonged immobility, nephrotic syndrome, paroxysmal nocturnal hemoglobinuria, obesity, and thyroid dysfunction.[75–85]

Testing for heritable thrombophilia in humans includes evaluation for deficiencies of AT, the natural anticoagulants protein C and protein S, the point mutations factor V Leiden and the prothrombin gene that result in procoagulant states.[86] Functional tests for protein S, protein C, and AT assess the activity level of each in plasma.[87–89] The screening test for factor V Leiden is the activated protein C resistance (APCR) test followed by PCR analysis if an abnormal APCR test is noted.[90] For detecting the prothrombin gene mutation, a multiplex PCR test is used as a screening test.[91] In humans, there is little evidence to suggest that inherited thrombophilia leads to development of arterial thrombophilia. Tests for inherited thrombophilia are not recommended in cases of myocardial infarction, stroke, or peripheral arterial thrombosis.[92] While inherited thrombophilias are not a common cause of stroke, patients with cryptogenic stroke generally undergo screening for inherited diseases, particularly the non-elderly.[93]

The diagnosis of antiphospholipid antibody syndrome, an acquired cause of thrombophilia, requires both laboratory and clinical abnormalities in accordance to the Sappora criteria.[94] The initial screening test for antiphospholipid antibody syndrome is most commonly performed with activated partial thromboplastin time (aPTT). If the aPTT is prolonged, the patient's plasma is mixed with normal plasma.[95] Addition of plasma with normal coagulation factor concentrations will be sufficient to overcome deficiency of any of these proteins, but not to reverse the effects of an inhibitor of coagulation. After mixing, if there is correction into the

normal range, this indicates a factor deficiency and the lack of correction suggests presence of an inhibitor. If the aPTT remains prolonged, the hexagonal phospholipid (HPPL) test is performed as a confirmatory test in which HPPL binds to lupus anticoagulant and thus the aPTT will shorten in the presence of the lupus anticoagulant.[95] Additional acquired thrombophilias including myeloproliferative neoplasms and paroxysmal nocturnal hemoglobinuria can be suspected due to identification of circulating neoplastic cells and formation of thrombosis at unusual sites.[96] Acquired decreases of AT, protein C, or protein S are more common than hereditary deficiencies and must be excluded. These proteins may be decreased in disseminated intravascular coagulation, liver disease, L-asparaginase therapy, or surgery.[87,88,97] Decreases of protein S and less often AT can be seen during pregnancy or estrogen use.[98–100] Acute phase reactions decrease free and functional protein S.[101] Proteinuria and heparin are associated with decreases in AT, while vitamin K antagonist therapy or vitamin K deficiency decreases protein C and protein S.[102]

A hypercoagulable state can be determined by measuring fibrin/fibrinogen degradation products, activation peptides, and enzyme-inhibitor complexes. A significant increase of prothrombin fragment 1+2, TAT, and fibrinopeptide A has been found in the elderly and with acute myocardial infarctions, hematologic malignancies, and pregnancy indicating activation of the coagulation pathways.[103] Increased factor VIII levels are associated with acute phase protein release and increased factor VIII activity is associated with a greater risk of venous thromboembolism (VTE).[104] Elevated activity levels of factor VIII associated with VTE risk seem to be not only due to acute phase response and appear to be persistent.[105] Short lived factor VIII levels in association with an acute phase protein release may be part of the

hypercoagulability seen with inflammatory disorders.[106] The adherence of platelets to damaged vessel walls is mediated by vWF and an elevated plasma level of vWF may predict occurrence of thrombosis.[107] A combination of coagulation and fibrinolytic measurements can be useful for determining a hypercoagulability in conditions that have a risk of thrombosis.

Clinically in veterinary medicine, there are few laboratory tests for hypercoagulability and those that exist are primarily aimed at detecting excessive thrombin generation. Viscoelastic-based testing methods are used to detect accelerated fibrin formation or strong fibrin clots. Thromboelastography (TEG) detects and provides documentation of the changes in viscoelastic properties of whole blood from the clot formation stage through fibrinolysis.[108] A second analyzer, rotational thromboelastometry (ROTEM), has also been described in veterinary medicine which measure the same variables, but utilize different terminology.[108] Validation for use of TEG and ROTEM have published for dogs, horses, and cats.[109–111] Hypercoagulability has been detected based on results of TEG tracings in dogs with disseminated intravascular coagulation, parvoviral enteritis, immune-mediated hemolytic anemia, and neoplasia.[112–116] Results of TEG have been variable in predicting thromboembolic risk in humans and more information is needed to determine the utility of this assay in dogs and cats at risk for thrombosis.[117]

C. Hyperthyroidism in humans and experimental animals and hypercoagulability

The first reports of an association between thyrotoxicosis and thrombosis were documented in 1913 and 1927 in people with cerebral venous thrombosis.[118,119] The cause of thrombosis in hyperthyroid humans is not completely understood, but likely a multifactorial

phenomenon. Multiple studies have indicated that hyperthyroid humans have increased risk for both venous and arterial thromboembolism (ATE).[120–122] In a large population-based case-control study of individuals with deep vein thrombosis and/or pulmonary embolism, it was concluded that elevated free thyroxine (T4) concentrations constitute a strong risk factor for venous thrombosis.[123] Within 3 months of the diagnosis of hyperthyroidism, the risk for a cardiovascular event is elevated, being highest for arterial embolism and atrial fibrillation. Although many case reports of ATE associated with hyperthyroidism have been reported, the relative risk of arterial embolism or stroke being unclear.[124,125] Risks of VTE, acute myocardial infarct, ischemic and non-ischemic stroke and percutaneous coronary intervention have also been reported to have a relative risk increased by 2- to 3-fold.[122] A retrospective cohort study identified an increased incidence of VTE in hyperthyroid people (8.7/1000) compared to the general population (0.6-1.6/1000).[126]

Alterations in the coagulation-fibrinolytic system in hyperthyroid people include increases of fibrinogen, FVIII, FIX, FX, vWF antigen, PAI-1, vWF type C domain (vWF:C), FVIII:C, vWF ristocetin cofactor, and plasma thrombomodulin, as well as decreases of protein C, protein S, prothrombin time (PT), aPTT, plasminogen, and tPA.[127–144] Hyperthyroidism leads to a hypercoagulable, hypofibrinolytic state, with opposite effects occurring in hypothyroid people.[121,145–147] A return to a euthyroid state corrects these alterations. Thus, it seems likely that alterations in hemostasis are due to the direct effects of thyroid hormones.

Thyroid hormones modulate gene transcription of coagulation proteins and protein synthesis of endothelial proteins. Elevated plasma levels of endothelium-derived proteins and peptides seen in hyperthyroidism can partially be explained by mRNA expression and protein

synthesis of vWF, fibronectin and endothelin-1 by protein kinase C α-, βII-, and ε-independent pathways caused by thyroid hormone-induced upregulation.[148] Triiodothyronine (T_3) administration to hypothyroid mice identified instant and lagged response of coagulation genes implying T_3 can either directly or indirectly control transcription and altered plasma levels of coagulation factors resulted from these transcriptional changes.[149] Induction of fibrinogen by T_3 has been demonstrated to be regulated largely at the level of transcription giving a understanding into the dysregulation of clotting profiles associated with hyperthyroidism.[150]

Altered clot structure and or lysis could be another explanation for higher thrombotic risk in hyperthyroidism. In a study investigating clot formation and lysis in hyperthyroidism, impaired fibrinolysis was identified.[151] By restoring euthyroidism, a partial normalization of clot structure/lysis was achieved. Changes in plasma coagulation factor levels were partly related to the mechanism of altered clot structure, but complement protein C3, the inflammatory pathway, was also implicated. More compact clots were seen with hyperthyroidism and also clots that were resistant to fibrinolysis ex vivo, which was related to the degree of hyperthyroidism and C3 levels. In the fibrin clot, C3 has been detected which may modulate recruitment of neutrophils to the site of injury and may influence clot lysis.[152] During fibrin network formation, C3 has been incorporated into the clot, particularly in pathological conditions, which influences the fibrinolytic process.[153]

Autoimmune disorders, including hyperthyroidism (Graves' disease), have been determined to be at an increased risk of venous thromboembolism. Patients admitted to the hospital with immune-mediated disease could be at an increased risk of VTE.[154] During the first year after admission to the hospital with an autoimmune disorder, the risk of pulmonary embolism has

been observed to be high and although the risk decreased over time, for up to 10 years, the overall risk remained.[155] Previously, inflammation and venous thromboembolism have been linked. Thrombotic responses are modulated by inflammation by suppressing fibrinolysis, downregulating anticoagulants, and upregulating procoagulants.[156] Due to the autoimmune nature of Grave's disease in people, this could be a contributing factor to the pathophysiology of thromboembolism formation. Feline hyperthyroidism is similar to another form of hyperthyroidism in people termed toxic nodular goiters (Plummer's disease) which does not involve autoimmunity in the pathogenesis. As such, in felines, it is likely the direct effects of thyroid hormones that contribute to alterations in hemostasis if this occurs.

Thyroid hormones have several effects on the vascular system and the heart with clinical manifestations of hyperthyroidism resulting from the ability of thyroid hormones to alter cardiovascular hemodynamics.[157] Tachycardia is a common sign of hyperthyroidism and results from an increase in sympathetic tone and decrease in parasympathetic tone.[158] Although sinus tachycardia is the most common rhythm disturbance, in 5 to 15 percent of humans with hyperthyroidism atrial fibrillation is present. The incidence of stroke in the presence of atrial fibrillation has been reported to be more than fivefold in euthyroid humans.[159–161] Cardiac output in hyperthyroidism is 50 to 300 percent higher than in normal subjects due to a an increase in resting heart rate, decrease in systemic vascular resistance, an increase in blood volume, and increases in left ventricular contractility and ejection fraction.[162,163] These cardiac changes may be contributing factors to embolism formation by high shear stress leading to endothelial injury.

Effects on the vascular endothelium have been demonstrated in hyperthyroidism. Neutrophil extracellular traps (NET) formation has become apparent as a trigger of thrombosis.[164–166] Neutrophils become stimulated by pathogens and release neutrophil elastase and DNA-histone complex into the extracellular space, referred to as NET.[164,165] Patients with hyperthyroidism are documented to have elevated NET formation.[167] Further involvement of the endothelium has been demonstrated with elevated markers of contact surface activation. In the contact system, negatively charged surfaces such as platelet polyphosphate, heparin, NETs, microparticles, collagen, misfolded proteins, RNA, and DNA can activate factor XII which converts prekallikrein (PK) to α-kallikrein, cleaving high-molecular-weight kininogen (HMWK) to produce bradykinin.[166] Factor XIIa activates factor XI and the intrinsic pathway. In hyperthyroid patients, elevations in PK, HMWK, factor XIIa and bradykinin were demonstrated, which are markers of contact surface activation.[167] Thyroid hormone receptor β (THRB) expression in endothelial cells has been previously documented.[168] Unaltered parameters of fibrinolysis and coagulation have been seen in a cohort of patients with resistance to thyroid hormone despite circulating elevated thyroxine suggesting procoagulant effects mediated via a THRB-dependent pathway showing further alteration of the vascular endothelium.[77] Endothelial associated proteins, including vWF, have been demonstrated to be increased in hyperthyroid humans. vWF is derived from the endothelium and serves as a marker of endothelial dysfunction. Thyroid hormones also alter gene expression of endothelial and hepatic derived proteins involved in endothelial function.[169] Additionally, vWF production can be increased in response to catecholamines. Thus, in hyperthyroid humans, increases in vWF is likely due to both the

effects of thyroid hormones on the endothelium as well as increased vWF synthesis. As a sequalae, a prothrombotic state can occur.

D. Thromboembolism formation in hyperthyroid cats

The mechanism of thrombus formation in hyperthyroid cats is unknown. Hyperthyroidism results in complex changes that alter circulation and cardiac structure and function. This thyrotoxic cardiac disease likely contributes to the development of TE in hyperthyroid cats. Cardiomyopathy can develop in hyperthyroid cats with echocardiographic findings of left ventricular and interventricular septal hypertrophy and increased left atrial diameter, although in more recent studies, the cardiac changes are usually mild.[170–172] Systemic hypercoagulability has been identified in cardiomyopathic cats.[173–176] This established hypercoagulability in cardiomyopathic cats could be a risk factor in development of ATE. A retrospective study of 100 cases of feline distal ATE identified echocardiographic evidence of left ventricular hypertrophy in most cats; 3% of the cohort was known to be hyperthyroid.[177] However, hyperthyroid cats with echocardiographically normal hearts have been documented to have episodes of ATE, suggesting there are other mechanisms for TE formation in hyperthyroid cats that are unrelated to structural cardiac disease.[178]

Additionally, a predisposition for thrombus formation can exist independently of left atrial enlargement as seen with hypertrophic cardiomyopathy (HCM), where left atrial size was not associated with laboratory evidence of hypercoagulability.[174] Structural changes in hearts of hyperthyroid cats are quite variable, but a previous study found that the cardiac biomarkers NT-pro brain natriuretic protein and troponin I used for identifying cats with hemodynamic

derangement or myocardial necrosis are indistinguishable in cats with hyperthyroidism or subclinical HCM, supporting the substantial effect of hyperthyroidism on the heart.[172]

As hypercoagulability occurs in hyperthyroid people, the role of the effects of hyperthyroidism on hemostasis independent of cardiomyopathy in cats could predispose to thrombosis. Little is known about the alterations in hemostasis in hyperthyroid cats. Thrombocytosis was found in 40% of hyperthyroid cats whereas another study found 14% of hyperthyroid cats had thrombocytopenia.[179,180] Platelet function was evaluated in 16 hyperthyroid cats using an analyzer (PFA-100) that stimulates primary hemostasis under high shear conditions similar to those in arterioles, and no significant difference in platelet function was seen in hyperthyroid and healthy age-matched control cats.[181] In a study comparing 11 cats with acquired heart disease, in which 9 had hyperthyroidism, to healthy cats, platelets from cats with acquired heart disease had decreased aggregation and serotonin release to adenosine diphosphate and increased responsiveness to collagen.[182]

A study describing the coagulation status of 15 hyperthyroid cats before and 14 days after radioiodine therapy compared with healthy age-matched controls found that PT was significantly shorter and fibrinogen concentration was significantly higher in hyperthyroid cats.[180] Results of TEG showed overall a hypercoagulable tendency, but only in some phases of the global coagulation process. Hyperthyroid cats with echocardiographic abnormalities were excluded to remove the influence of secondary cardiomyopathy on coagulation. These findings support that thyroid hormones in cats affect hemostasis markers and that they can in some cases promote a hypercoagulable state. In the aforementioned study, some changes remained after a normal serum T4 was documented following RIT, namely increased fibrinogen

concentration, shortened PT, and an increase in TEG variables reflecting overall clot stability. This was suggested to occur as a result of radiation-induced thyroiditis and induction of an acute-phase protein response. Further studies assessing hemostasis in hyperthyroid cats before and after treatment are indicated in addition to identification of any long-term consequences (e.g. thrombosis formation).

CHAPTER 3: EVALUATION OF HEMOSTASIS IN HYPERTHYROID CATS

A. Introduction

Feline ATE is a painful and often fatal condition in cats; less than 50% of cats survive to discharge due largely to the decision for humane euthanasia.[177] For those that do survive, long-term prognosis is guarded with recurrence rates of 45-75%.[177,183,184] Risk factors for thromboembolism (TE) include cardiomyopathies, neoplasia, and hyperthyroidism.[178] Two retrospective studies have revealed that as many as 9% of cats that were presented for management of ATE were hyperthyroid.[178,185] In the evaluation of 16 cats with confirmed via histology cerebrovascular disease, 3 of these cats had hyperthyroidism.[186]

Mechanisms of thrombus formation are multifactorial and include abnormal blood flow, endothelial damage, and hypercoagulability.[187] In cats with cardiomyopathy, thrombus formation has been attributed to blood stasis, endothelial injury, and possibly hyperactive platelets.[175,188,189] There is some evidence to suggest cats with cardiomyopathy might also be hypercoagulable.[173,174] The mechanism of thrombus formation in hyperthyroid cats is unknown. Hyperthyroidism results in complex changes that alter circulation and cardiac structure and function. The resultant thyrotoxic cardiac disease likely contributes to the development of TE in

hyperthyroid cats.[171] However, hyperthyroid cats with echocardiographically normal hearts have been documented to have episodes of ATE. A predisposition for thrombus formation can exist independently of structural abnormalities such as atrial enlargement.[174] This is consistent with the existence of mechanisms other than cardiac disease that might be responsible for TE formation in hyperthyroid cats.[178] Structural changes in the hyperthyroid heart are quite variable, but we have previously documented that the cardiac biomarkers NT-pro brain natriuretic factor and troponin I are indistinguishable in cats with hyperthyroidism or HCM, supporting a substantial effect of hyperthyroidism on the heart.[172]

Thromboembolism also occurs in people with hyperthyroidism.[127,190–194] Hyperthyroid humans are 2 times more likely to develop TE compared to euthyroid counterparts.[194,195] Thyroid dysfunction alters primary and secondary hemostasis to create a hypercoagulable and hypofibrinolytic state. Primary hemostasis is affected by increased vWF while secondary hemostasis is affected by increased coagulation proteins such as factor VIII, factor IX, and fibrinogen, and impaired fibrinolysis is indicated by increased plasminogen activator inhibitor.[123,195–197]

Hyperthyroidism also leads to endothelial dysfunction which contributes to a hypercoagulable state in people.[198] Hyperthyroid cats have hypercoagulable tendencies as indicated by hyperfibrinogenemia and shortened PT which might explain the occurrence of TE events in affected cats that do not have clinically detectable thyrotoxic heart disease.[180]

The purpose of this study is to evaluate markers of hypercoagulability in hyperthyroid cats as compared to healthy controls and after treatment with RIT. Our hypotheses were that

hyperthyroid cats would have altered hemostatic markers and that there would be resolution of this altered hemostasis post RIT.

B. Materials and Methods

a. Animals

Cats referred to the Virginia-Maryland College of Veterinary Medicine (VMCVM) for radioiodine treatment of hyperthyroidism were eligible for enrollment. Hyperthyroidism was defined as a serum T4 concentration that exceeded the upper limit of the reference interval (T4 > 37.7 nmol/L) as well as compatible findings on history (e.g., polyphagia, weight loss, polyuria, polydipsia, hyperactivity) and physical examination (e.g., presence of thyroid nodule, poor body and muscle condition scores). Cats were excluded if they received methimazole or a low-iodine diet in the 2 weeks prior to presentation. In addition, cats were excluded if they received medications known to alter hemostatic parameters (e.g., prednisone, aspirin, clopidogrel, heparin) or had evidence of concurrent illness outside of the consequences of hyperthyroidism based on lack of significant abnormalities on routine physical examination, a complete blood count, serum or plasma biochemistry profile, TSH, and urinalysis. VMCVM staff- and student-owned cats deemed healthy based on routine physical examination, a complete blood count, serum or plasma biochemistry, serum T4, and urinalysis made up the control group.

b. Markers of Hypercoagulability

Approximately 2.7mL of blood was collected into a syringe containing 0.3mL of citrate by jugular venipuncture. Blood samples were excluded if citrated samples contained clots or if there was traumatic venipuncture. The blood was transferred into a plastic tube and

centrifuged for 10 minutes at 5°C and 1,500 x *g*. The plasma was transferred to a polyethylene tube and stored at -70°C until shipped for batch analysis (samples sent approximately every 4-6 months) to the Comparative Coagulation Laboratory at Cornell University College of Veterinary Medicine. Plasma was analyzed for PT, aPTT, concentrations of fibrinogen, D-dimers, AT, TAT, vWF:Ag, and activity of factors VIII and IX. Hypercoagulability was defined based on a previous study[173] as 2 or more of the following abnormalities in hyperthyroid cats relative to the reference intervals: decreased AT activity, increased factor VIII or IX activities, or increased concentrations of TAT, fibrinogen, or D-dimers. These tests were repeated between 6 to 9 months post RIT.

c. Echocardiography

A routine echocardiogram was performed at the time of enrollment in all cats. The following variables were obtained from M-mode and two-dimensional (2D) echocardiograms. Normal echocardiographic findings were defined by: left ventricular free wall and interventricular septal wall thickness at end-diastole of <6mm, 2D left atrium to aorta ratio (LA:Ao) of $\leq$ 1.5, no or only trivial (clinically insignificant) insufficiencies of the pulmonic and tricuspid valves, and no insufficiency of the aortic and mitral valves. Cats screened for inclusion as healthy controls were excluded if echocardiographic abnormalities were noted. Hyperthyroid cats with echocardiographic abnormalities remained in the study. All echocardiograms were performed by the same operator to reduce measurement variation related to operator. The echocardiographic dimensions were measured by a different cardiologist who was unaware of which group the cat belonged to.

Hyperthyroid cats were treated with I-131 and, if documented to have resolution of hyperthyroidism based on serum T4 6 months or more after treatment and available for re-evaluation, underwent assessment of coagulation and echocardiographic evaluation identical to that prior to treatment.

d. Statistical analysis

Statistical analyses were performed using commercially available computer software. A power analysis showed that 13 cats per group (hyperthyroid and healthy controls) would be needed to detect a difference of 0.40 in the proportions of cats with a hypercoagulable state with a power of 82.8%. The power analysis was calculated with an expected 40% prevalence of hypercoagulability in the affected group and a 0% prevalence in the control group. Predictions for the prevalence of hypercoagulability were made based on a previous study evaluating hypercoagulability in cats with cardiac disease.[173] Hyperthyroid cats were grouped based on the presence or absence of structural cardiac abnormalities to compare significant differences in hypercoagulability and, thus, determine if cardiac changes may contribute to any alteration of hemostasis.

The proportion of cats with hypercoagulable states were compared between the two groups (hyperthyroid and control) using Fisher's exact test. For continuous variables, normal probability plots were used to determine whether the distribution of the data was approximately normal. If data were normally distributed, a two-sample t-test was used to compare coagulation variables (PT, aPTT, fibrinogen, D-dimers, AT, TAT, vWF:Ag, and factors VIII and IX) between healthy and hyperthyroid cats as well as between hyperthyroid cats with or without structural cardiac abnormalities. For continuous data that were not normally

distributed, comparisons were made using a Wilcoxon rank sum test. Additional analyses were performed to determine if the resulting groups (hypercoagulable vs non-hypercoagulable vs controls) differed with respect to serum T4, duration of hyperthyroidism, and left atrial size. If data were normally distributed, a one-way analysis of variance (ANOVA) model was performed followed by contrasts to extract comparisons of interest. For skewed data, a Kruskal-Wallis test was performed followed by a series of Wilcoxon rank sum tests.

For normally distributed data, a paired t-test was used to compare coagulation variables before and after treatment in hyperthyroid cats. For skewed data, a Wilcoxon signed rank test was used.

An alpha value of 0.05 was set for all tests.

C. Results

a. Study population

Twenty-five hyperthyroid cats met the inclusion criteria and were enrolled in the study. The hyperthyroid cats ranged in age from 10 to 16 years (median, 13.0 years). Thirteen clinically normal euthyroid cats were enrolled as controls. The control cats ranged in age from 8 to 16 years (median, 12.0 years). The hyperthyroid group was composed of 13 spayed females and 12 neutered males and the euthyroid group was composed of 9 spayed females and 4 neutered males. Breeds from both groups included domestic longhair and shorthair (38 cats), Himalayan (2 cats), Siamese (1 cat), and Burmese (1 cat). Body weights of the hyperthyroid cats ranged from 2 to 6.3 kg (median, 4 kg) and the body weights of the euthyroid control cats ranged from 3.1 to 6.4 kg (median, 4.9 kg).

Of the original 25 hyperthyroid cats, 9 underwent for recheck evaluation between 6-9 months post-RIT. Ten of the initial hyperthyroid cats were lost to follow-up, 3 were euthanized prior to the recheck time frame, 2 were hyperthyroid based on serum T4 at 6 months post-RIT, and 1 was not treated with RIT.

b. T4 and TSH concentrations

The T4 concentration in the hyperthyroid cats ranged from 49 to 509 nmol/L (median, 130 nmol/L [reference interval (RI) 16.0-37.7 nmol/L]) and TSH concentration ranged from 0.02 to 0.1 ng/mL (median 0.02 ng/mL [RI 0.021-0.124 ng/mL]). The T4 concentration in the euthyroid control cats ranged from 19.4 to 34.9 nmol/L (median, 28.4 nmol/L).

Post radioactive iodine, 9 cats presented for recheck evaluation and 2 were persistently hyperthyroid and were excluded from analysis. Serum T4 concentration was normal in 7 cats, while 2 of the 7 had a serum TSH concentration >0.3 ng/mL. Of the 7 post RIT cats, the T4 concentration ranged from 16.3 to 33.5 nmol/L (median 18.4 nmol/L) and the TSH concentration ranged from 0.02 to 2.87 ng/mL (median 0.076 ng/mL).

c. Echocardiography

Based on the established criteria, 19/25 (76%) hyperthyroid cats had abnormal echocardiographic findings. In the assessment of left atrial size, no correlation was noted between serum T4 level and LA:Ao ratio.

The presence of an abnormal echocardiogram in hyperthyroid cats was associated with significantly higher median fibrinogen concentration in comparison to presence of a normal echocardiogram in hyperthyroid cats (Table 1). A normal or abnormal echocardiogram finding

did not have a significant impact on the remaining hemostatic markers in hyperthyroid cats (Table 1).

Seven cats in the hyperthyroid group had echocardiography performed after RIT and documentation of a normal serum T4 concentration. Six of the seven cats had abnormal echocardiograms on initial presentation. Only one of these 6 cats had resolution of the echocardiographic changes after treatment. No significant difference was appreciated in evaluation of LA:Ao ratio from initial evaluation to recheck.

d. Markers of hypercoagulability

Based on our definition of hypercoagulability, the prevalence of hypercoagulability was higher (P = 0.019) among hyperthyroid cats was 70.8% (17/24) in comparison to the prevalence among euthyroid control cats of 30.8% (4/13). One hyperthyroid cat was excluded from this analysis due to lack of Factor VIII and Factor IX information. The serum T4 concentration was significantly associated with hypercoagulability (P = 0.043). In regards to the each hemostatic variable, 36% (9/25) of hyperthyroid cats and 38% (5/13) of control cats had decreased aPTT, 56% (14/25) of hyperthyroid cats had increased fibrinogen, 32% (8/25) of hyperthyroid cats and 23% (3/13) had increased d-dimers, 40% (10/25) of hyperthyroid cats and 15% (2/13) of control cats had increased vWF:Ag, 40% (10/25) of hyperthyroid cats and 15% (2/13) of control cats had increased TAT, 20% (4/20) of hyperthyroid cats and 23% (3/13) of control cats had increased factor VIII, 30% (6/20) of hyperthyroid cats and 30% (4/13) of control cats had increased factor IX (Table 2). The mean number of hemostatic variables that support a hypercoagulable state in hyperthyroid cats was 2.26 (range 0-6), while the mean number for

control cats was 1.86 (range 0-5). Of the hypercoagulable cats 60% (13/21) had abnormal echocardiograms.

Of the 6 cats in the hyperthyroid group that had hemostasis testing repeated, 5 initially were determined to be in a hypercoagulable state that resolved after RIT in 2. Four of these 5 cats had abnormal echocardiograms on initial presentation. After resolution of hyperthyroidism, 2 cats had resolution of a hypercoagulable state with one of the cats on initial presentation having a normal echocardiogram before and after RIT and the other cat having a persistently abnormal echocardiogram. Three cats had persistence of the defined hypercoagulable state and concurrent abnormal echocardiograms. One hyperthyroid cat with an abnormal echocardiogram on presentation and at recheck did not meet the study criteria for hypercoagulability at either time point.

Hyperthyroidism was associated with a higher median fibrinogen concentration than euthyroid controls (Figure 1). Once hyperthyroidism was resolved after RIT, the fibrinogen concentration was decreased (Figure 2). Hyperthyroidism was also associated with a higher median AT activity than euthyroid controls (Figure 3). Post RIT, the AT activity was significantly decreased (Figure 4). The hyperthyroid group also had a significantly higher median vWF:Ag concentration in comparison to euthyroid controls (Figure 5). Post RIT, the median vWF:Ag concentration was significantly decreased (Figure 6). There was no significant difference in D-dimer concentrations between hyperthyroid cats and euthyroid controls, but post RIT, the D-dimer concentration was significantly increased in comparison to initial evaluation in hyperthyroid cats (Figure 7).

For the remaining markers of hemostasis in hyperthyroid cats (PT, aPTT, TAT, Factor VIII, Factor IX), there was no significant difference when compared to euthyroid controls or post RIT values (Tables 3 and 4). Serum T4 concentration was positively correlated with fibrinogen and AT concentrations (Figures 8 and 9).

Sedation did not have a significant impact on any hemostatic variable (Table 5).

D. Discussion

The majority of hyperthyroid cats in this study were considered hypercoagulable. In addition, hyperthyroid cats had significantly higher vWF than euthyroid controls. In hyperthyroid cats with abnormal echocardiograms, only fibrinogen was significantly higher compared to hyperthyroid cats with normal echocardiograms. It appears that hyperthyroid cats can have altered hemostasis and hypercoabulability independent of thyrotoxic cardiomyopathy or concurrent primary hypertrophic cardiomyopathy.

Our definition of hypercoagulability was adopted from Stokol et al which evaluated hypercoagulability in cats with primary HCM.[173] This definition was used to identify coagulation factor excess (fibrinogen, factor VIII and factor IX), increased thrombin generation (increased TAT and D-Dimers), and decreased AT activity that could result in a hypercoagulable state. Based on this study, hypercoagulability in hyperthyroid cats could be associated with increased coagulation factor excess and increased thrombin generation.

Surprisingly, 30% of euthyroid control were determined to be hypercoagulable. Our control population consisted of cats $\geq$ 8 years of age in order to create an age-matched comparison to the hyperthyroid cat group since hyperthyroidism occurs mostly in older cats. In creating an older control group population, there is the risk of enrolling cats with other

underlying conditions. Screening of the control group consisted of routine physical examination, a complete blood count, serum or plasma biochemistry, serum T4, and urinalysis in order to exclude this risk. There is the potential of underlying disease processes that were missed in our older control group since imagining such as abdominal ultrasound or thoracic radiography was not utilized.

Interestingly, an increase in AT activity was noted in hyperthyroid cats. This is in contrast to what would be expected in a hypercoagulable state in which there should be a decrease in AT activity. Theoretically, with an elevated AT activity, a patient could be at risk for bleeding tendencies. Currently there is no evidence to support that an elevation in AT activity leads to bleeding tendencies and the clinical relevancy in elevated activity of AT seen in this population of hyperthyroid cats is unknown.

AT activity previously measured in cats with acquired heart disease showed an increased activity of AT in comparison to healthy cats. This increased activity was attributed to the fact this protein behaves as an acute-phase reactant.[182] Along with AT, fibrinogen is also a positive acute phase protein in response to inflammatory cytokines (IL-1, IL-6, TNFα). In this study and in a previous study of hyperthyroid cats[180], fibrinogen concentration is elevated which is in agreement with findings in hyperthyroid humans. Also seen in this study, there is a strong correlation of both AT and fibrinogen seen with serum T4. Serum amyloid A is another positive acute phase protein that has been documented to be elevation in feline hyperthyroidism,[199] although no increase was observed in a more recent study.[200] Serum amyloid A was not evaluated in this study. In humans, vWF:Ag has been described as an acute phase reactant that strongly correlated with elevations of serum C-reactive protein and normalized over time.[201]

Feline hyperthyroidism has been described histologically as occurring most commonly due to follicular cell adenoma and multinodular adenomatous hyperplasia, in contrast to the autoimmune hyperthyroidism described in humans,[202] with no increases in inflammatory cytokines leading to increases of acute-phase proteins described.

Another explanation for the increases of fibrinogen concentration and AT activity seen in hyperthyroid cats could be increased synthesis from the liver driven by hyperthyroidism. In the studies on influence of hormones on anti- and coagulant protein production by hepatocytes, thyroid hormone had an effect on the increase of antithrombin and fibrinogen based on evaluation of enrichment in culture mediums.[203] In hyperthyroid humans, increases in fibrinogen and AT activities have been observed and have been theorized to be effect of thyroid hormones on the liver reflecting the in vitro data described previously of thyroid hormones influencing the synthesis and secretion of various proteins of hepatic origin.[129]

D-dimer concentration was seen to be elevated post-RIT in this study which was unexpected. In hyperthyroidism there is an elevated metabolic rate which can lead to an increase of activity by hepatocytes. Post-RIT, with a reduction of the metabolic rate once thyroid hormone level decreases, less hepatic clearance of D-dimers by hepatocytes may be an explanation of elevation in this concentration.[204] Hyperthyroidism is thought to result in less fibrinogenolysis and fibrinolysis. D-dimers are degradation products of plasmin-cleaved cross-linked fibrin and are considered to be a more sensitive indicator of fibrinolysis. It could be post-RIT that a rebound fibrinolysis occurs upon normalization of serum T4.

The majority of hyperthyroid cats (76%) had abnormal echocardiograms with mostly mild cardiac changes. The prevalence of echocardiographic abnormalities is higher than

previously reported 37% of hyperthyroid cats prior to oral radioiodine administration.[205] Also in the referenced study, after 2-3 months, 32% had abnormal echocardiographic variables with some of these changes emerging after treatment. In our study, only 1 cat had a change from an abnormal to normal echocardiogram upon achieving resolution of hyperthyroidism with no cat changing from a normal to abnormal echocardiogram. This persistence in abnormal echocardiographic findings could be due to underlying cardiac disease unrelated to hyperthyroidism, permanent hyperthyroid-related damage, or longer than 6-9 months is necessary for thyrotoxic cardiomyopathy to reverse.

In this study, the left atrium size, based on the LA:Ao ratio, did not correlate with the serum T4 level. The absence of increased left atrial size seen in hyperthyroid cats noted in this study agrees with more recent research in which mostly mild cardiac changes were seen in hyperthyroid cats. This could be due to earlier identification of hyperthyroidism in this population or that the majority had previously received methimazole (16/25; 64%) for hyperthyroid management prior to presentation for RIT, although it was discontinued in the 2 weeks prior to presentation. Also, the number of cats achieving euthyroidism with methimazole prior to evaluation was unknown.

The fibrinogen concentration was higher in hyperthyroid cats with abnormal echocardiograms than those with normal echocardiograms. Although these hyperthyroid cats with abnormal echocardiograms showed a significant difference in the hemostatic marker of fibrinogen, the rest of the hemostatic parameters evaluated were normal. The prevalence of cardiac abnormalities in hyperthyroid cats was high with 19 cats having abnormal echocardiogram and only 6 had normal echocardiograms. In order to have a clearer picture of

the effect hemostatic markers, a euthyroid HCM group may be helpful in comparing these variables for future research. Given that hyperfibrinogenemia was found in cats with cardiomyopathies and our findings were associated with cardiac abnormalities, it would appear that hyperfibrinogenemia could be driven by cardiac changes. Hyperfibrinogenemia occurred in 37% of cardiomyopathic cats in a study evaluating hemostatic parameters in cats with cardiomyopathy.[173] Although all cats in the cardiomyopathy group in this study had left atrial enlargement. Overall, it would appear that the hypercoagulability seen in hyperthyroid cats is not solely attributed to cardiac abnormalities, but further evaluation would be required in order to assess if the fibrinogen elevation is independent of cardiac status.

Concentration of vWF:Ag was significantly higher in hyperthyroid cats compared to euthyroid controls. This could indicate endothelial injury or dysfunction in hyperthyroid cats. Endothelial injury along with or independent of hypercoagulability could promote a prothombotic state. In hyperthyroid humans, thyroid hormones can lead to upregulation of protein synthesis of endothelial proteins as well and enhanced adrenergic stimulation. These could be other reasons for a finding higher vWF:Ag concentration in our hyperthyroid cats. Cats with cardiomyopathy without ATE and those with ATE were found to have normal and increased vWF:Ag, respectively.[173] The cause of increase vWF:Ag in cats with ATE was thought to be due to endothelial injury from the thrombus, but prethrombotic endothelial injury could also be a possibility. Whether hyperthyroid cats have endothelial dysfunction and cats with ATE have endothelial injury prior to thrombosis requires further study. Other endothelium-derived proteins, such as tissue plasminogen activator and plasminogen activator inhibitor 1 would further help elucidate the role of the endothelium in hyperthyroid cats. Based on our study and

previous studies, it appears that cardiomyopathy is not the reason for the increased vWF:Ag concentration.

A limitation of this study was the small number of cats presenting for recheck evaluation with only 7 cats with a normal serum T4 undergoing follow-up testing. A possible limitation of this study is that 2 cats were subclinically hypothyroid post-RIT based on results of a normal T4 and an elevated TSH. As in humans, it is possible that a subclinical hypothyroid state could affect hemostasis and cardiac function.[206,207] Hypothyroidism can promote a hypocoagulable state in people. In cats, overt hypothyroidism can affect cardiac function, renal function, and overall survival. Only renal function has been evaluated in subclinical hypothyroid cats which appears to be decreased. Given that the effects of subclinical hypothyroidism on hemostasis are unknown and the main goals were to evaluated how these values change after resolution of a hyperthyroid state, we did not exclude subclinical hypothyroid cats. Whether subclinical hypothyroidism impacts hemostasis in cats requires further study.

CHAPTER 4: CONCLUSIONS AND FURTHER RESEARCH

The results of this study provide evidence of altered hemostasis and hypercoagulability and these alterations resolved after radioiodine therapy. Altered hemostasis and hypercoagulability in hyperthyroid cats that do not appear to be solely attributed to cardiac status. Coagulation factor excess, increased thrombin generation, and endothelial dysfunction are other mechanisms why these abnormalities occur in hyperthyroid cats.

Our study was unique in that multiple parameters of hemostasis were evaluated along with cardiac status in hyperthyroid cats. This study also reassessed these parameters of hemostasis post-RIT at a longer time interval than has previously been evaluated.

Some cats remained hypercoagulable despite resolution of hyperthyroidism. Further studies with a larger sample size of feline patients post-RIT could be necessary to rule out Type II error within this study.

In assessment of cardiac status of hyperthyroid cats in this population, the serum T4 elevation was not associated with left atrial size, which may support that the early identification of hyperthyroidism and early management combats the severity of thyrotoxic cardiomyopathy.

Continued investigations are needed to further characterize the hypercoagulable state and the long-term consequences as well as if endothelial injury occurs secondary to hyperthyroidism in cats.

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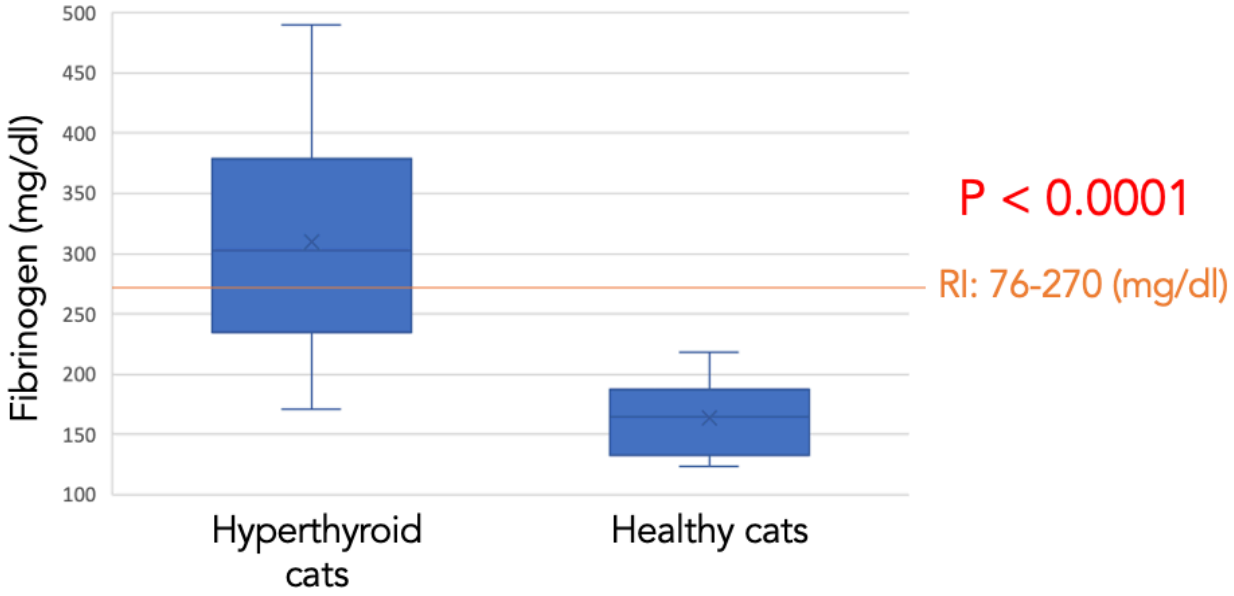
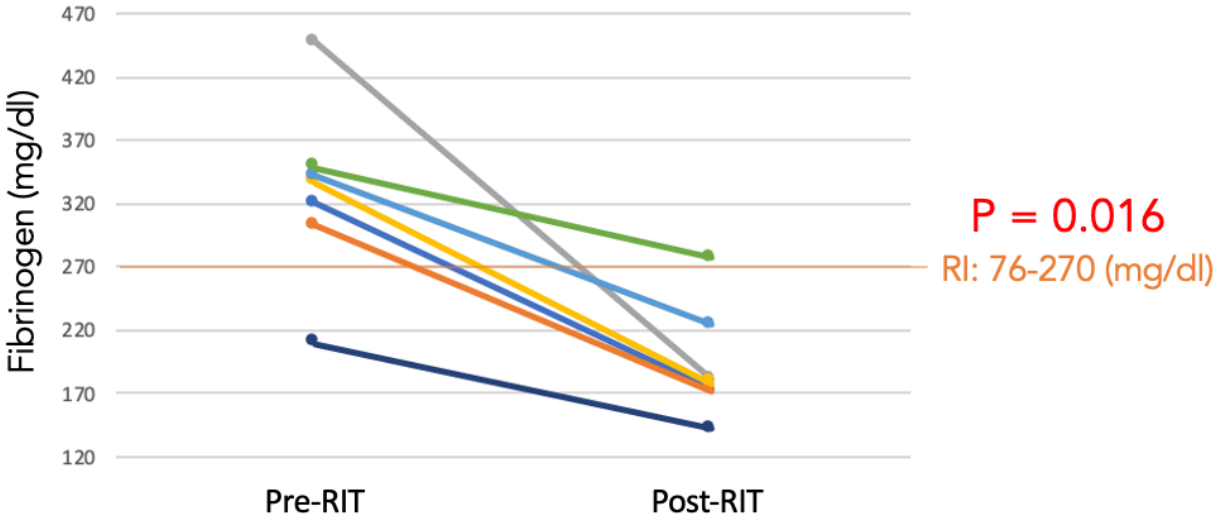
APPENDIX A: FIGURES

Figure 1. Fibrinogen concentration in hyperthyroid and euthyroid controls



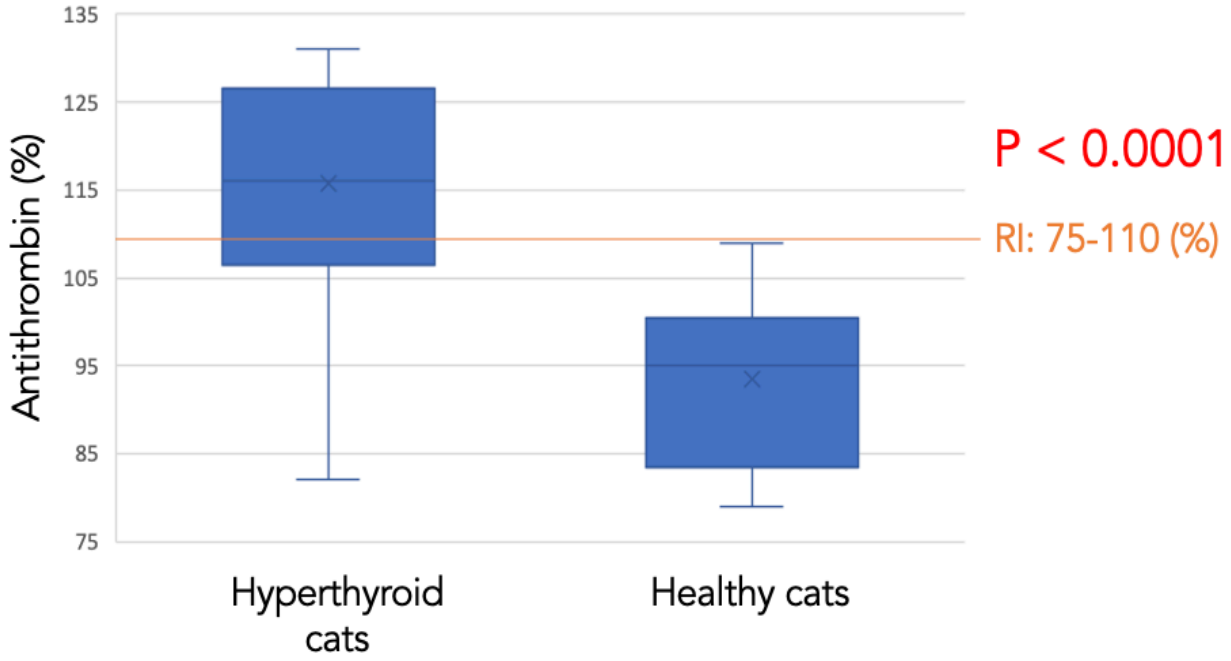
Hyperthyroidism was associated with a significantly higher median plasma fibrinogen concentration (n=25; 303 mg/dL; range 171-490 mg/dL) in comparison to euthyroid controls (n=13; 164 mg/dL; range 123-218 mg/dL). P-value was obtained by Mann-Whitney U test. A p-value <0.05 was considered significant.

Figure 2. Fibrinogen concentration in hyperthyroid cats pre- and post-RIT



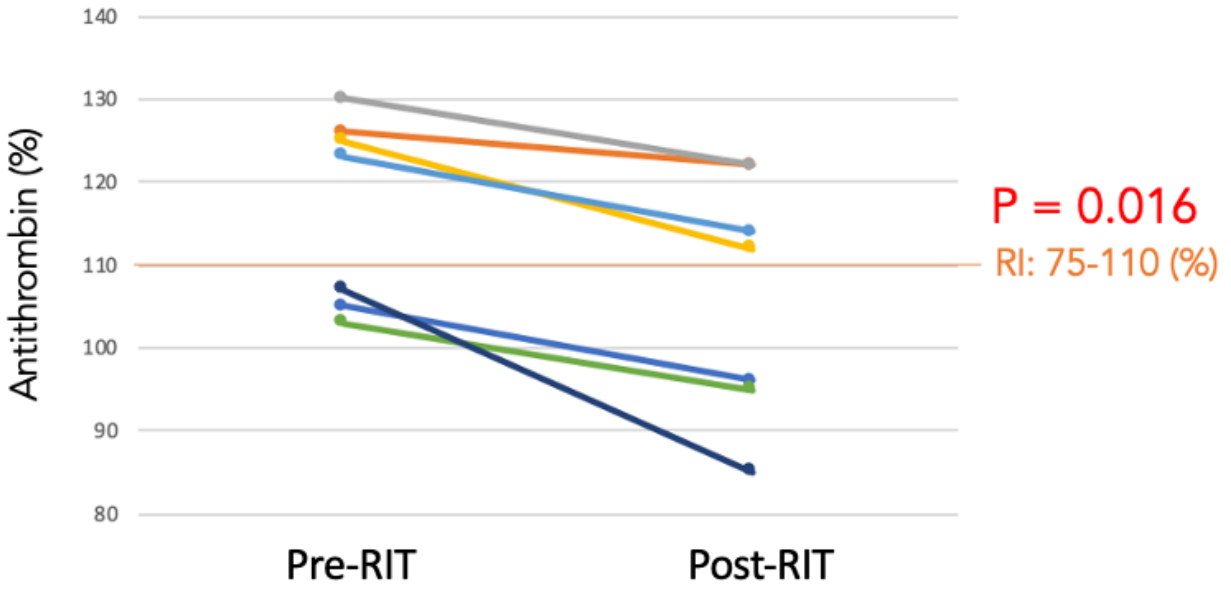
The median plasma fibrinogen concentration was significantly decreased post-RIT (n=7; median 178 mg/dL; range 142-277 mg/dL). P-value was obtained by Wilcoxon signed-rank test. A p-value <0.05 was considered significant.

Figure 3. Antithrombin activity in hyperthyroid and euthyroid controls



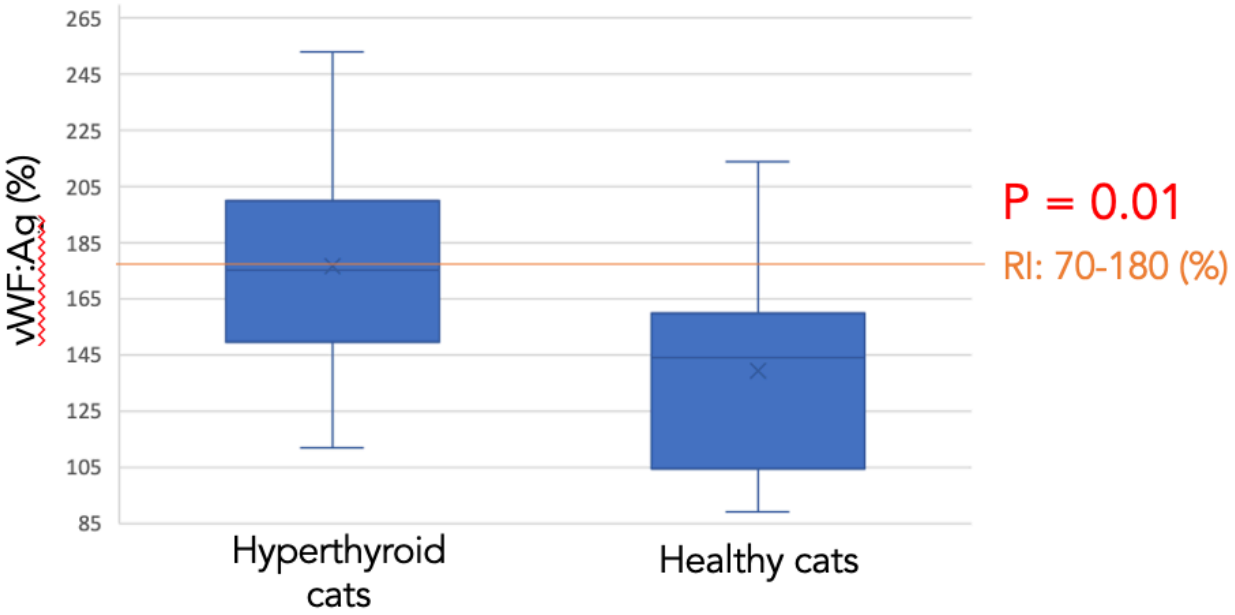
Hyperthyroidism was associated with a significantly higher median plasma antithrombin activity (n=25; 116%; range 82-131%) in comparison to euthyroid controls (n=13; 95%; range 79-109%). P-value was obtained by Mann-Whitney U test. A p-value <0.05 was considered significant.

Figure 4. Antithrombin activity in hyperthyroid cats pre- and post-RIT



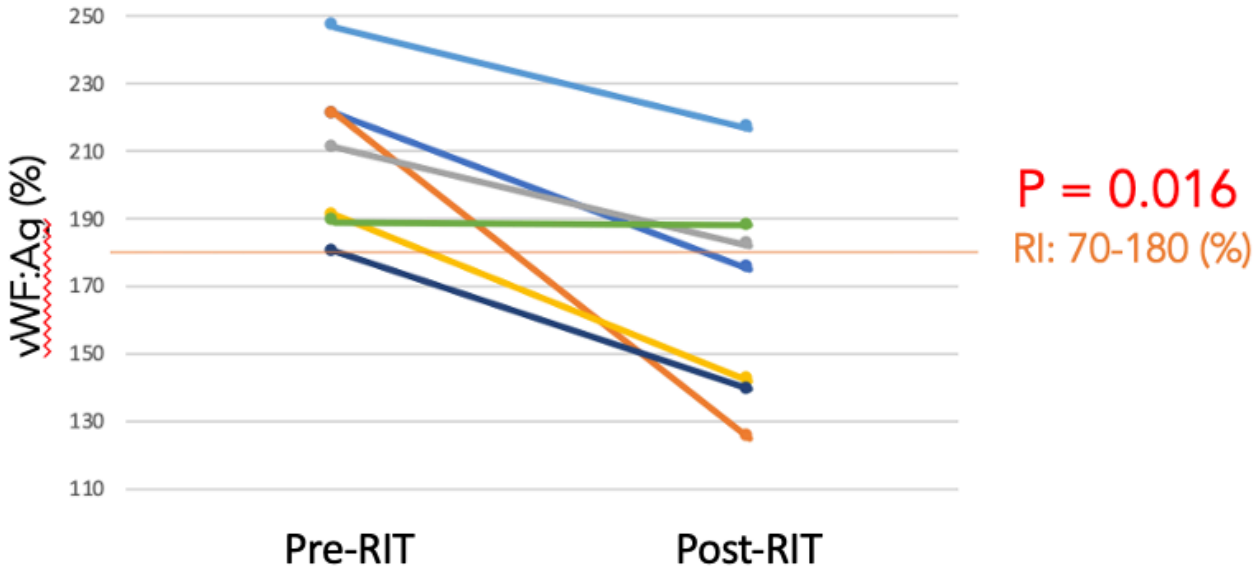
The median plasma antithrombin activity was significantly decreased post-RIT (n=7; median 112%; range 85-122%). P-value was obtained by Wilcoxon signed-rank test. A p-value <0.05 was considered significant.

Figure 5. Von Willebrand Factor antigen concentration in hyperthyroid and euthyroid controls



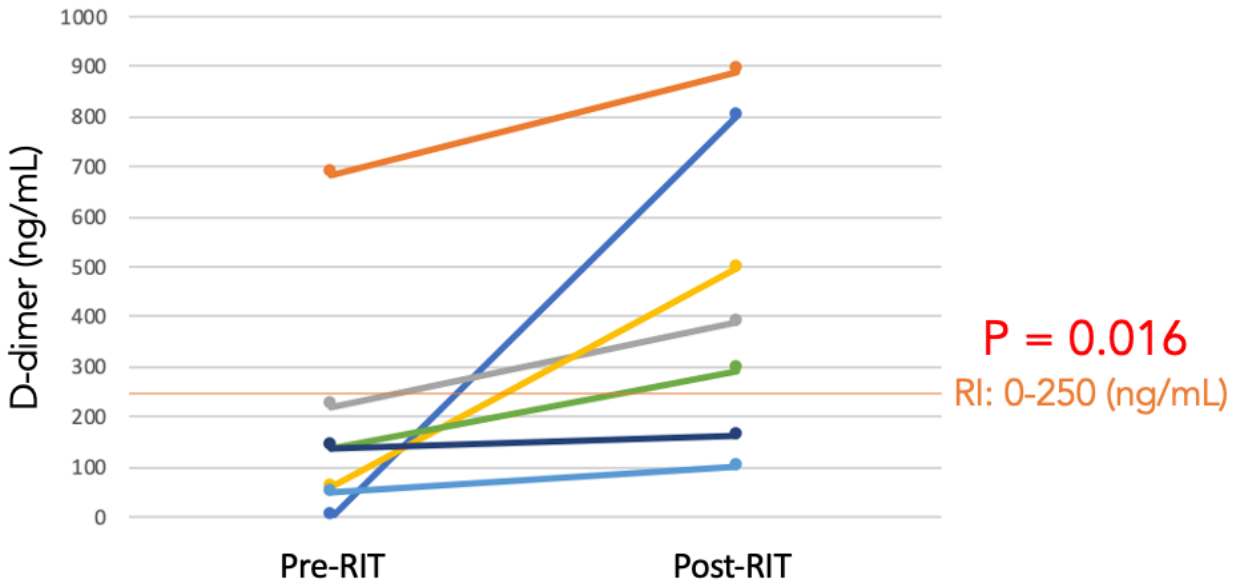
Hyperthyroidism was associated with a significantly higher median plasma vWF:Ag concentration (n=25; median 175%; range 112-253%) in comparison to euthyroid controls (n=13; 144%; range 89-214%). P-value was obtained by Mann-Whitney U test. A p-value <0.05 was considered significant.

Figure 6. Von Willebrand factor antigen level in hyperthyroid cats pre- and post-RIT



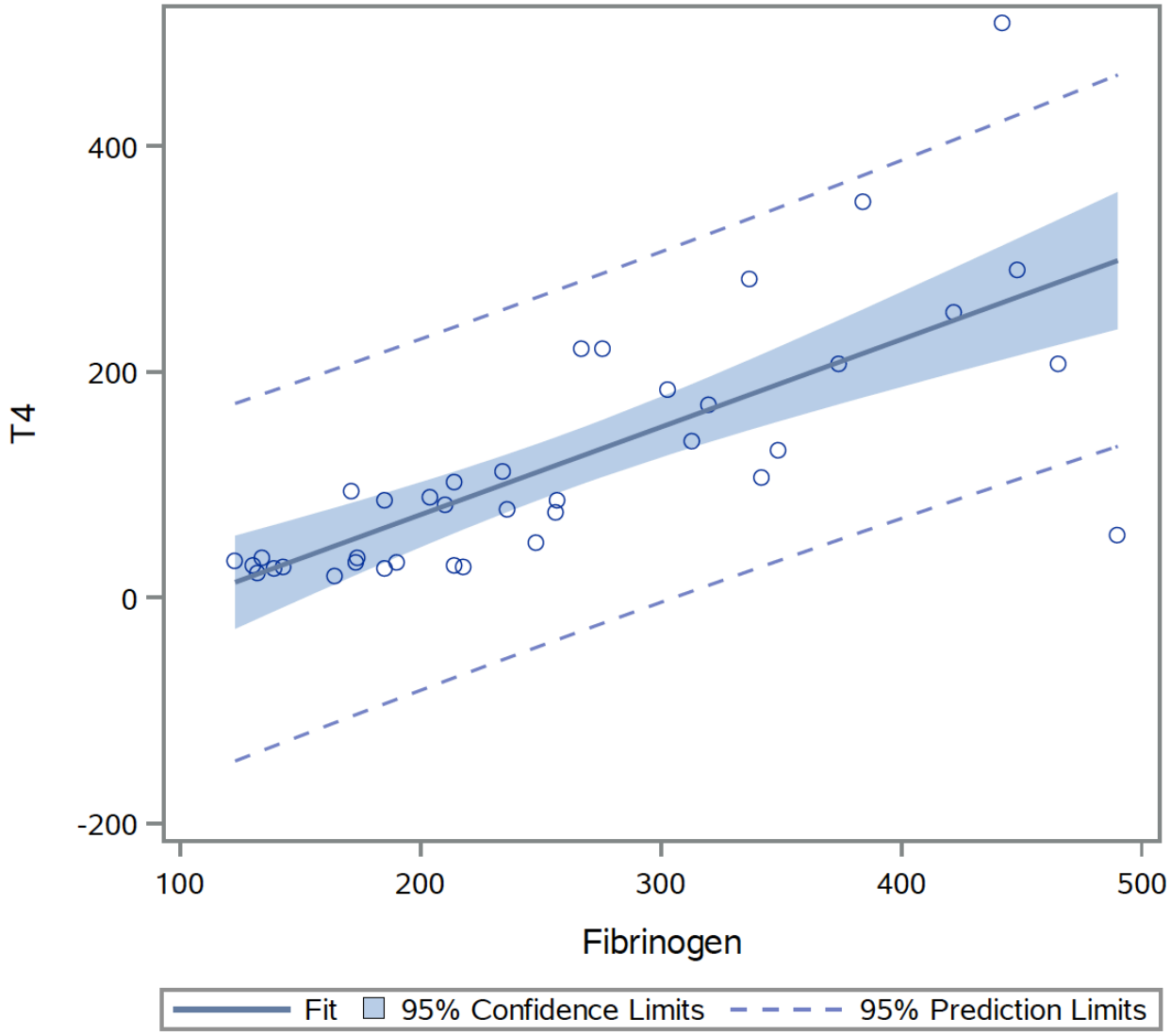
The median plasma von Willebrand factor antigen concentration was significantly decreased post-RIT (n=7; median 175%; range 125-217%). P-value was obtained by Wilcoxon signed-rank test. A p-value <0.05 was considered significant.

Figure 7. D-dimer concentration in hyperthyroid cats pre- and post-RIT



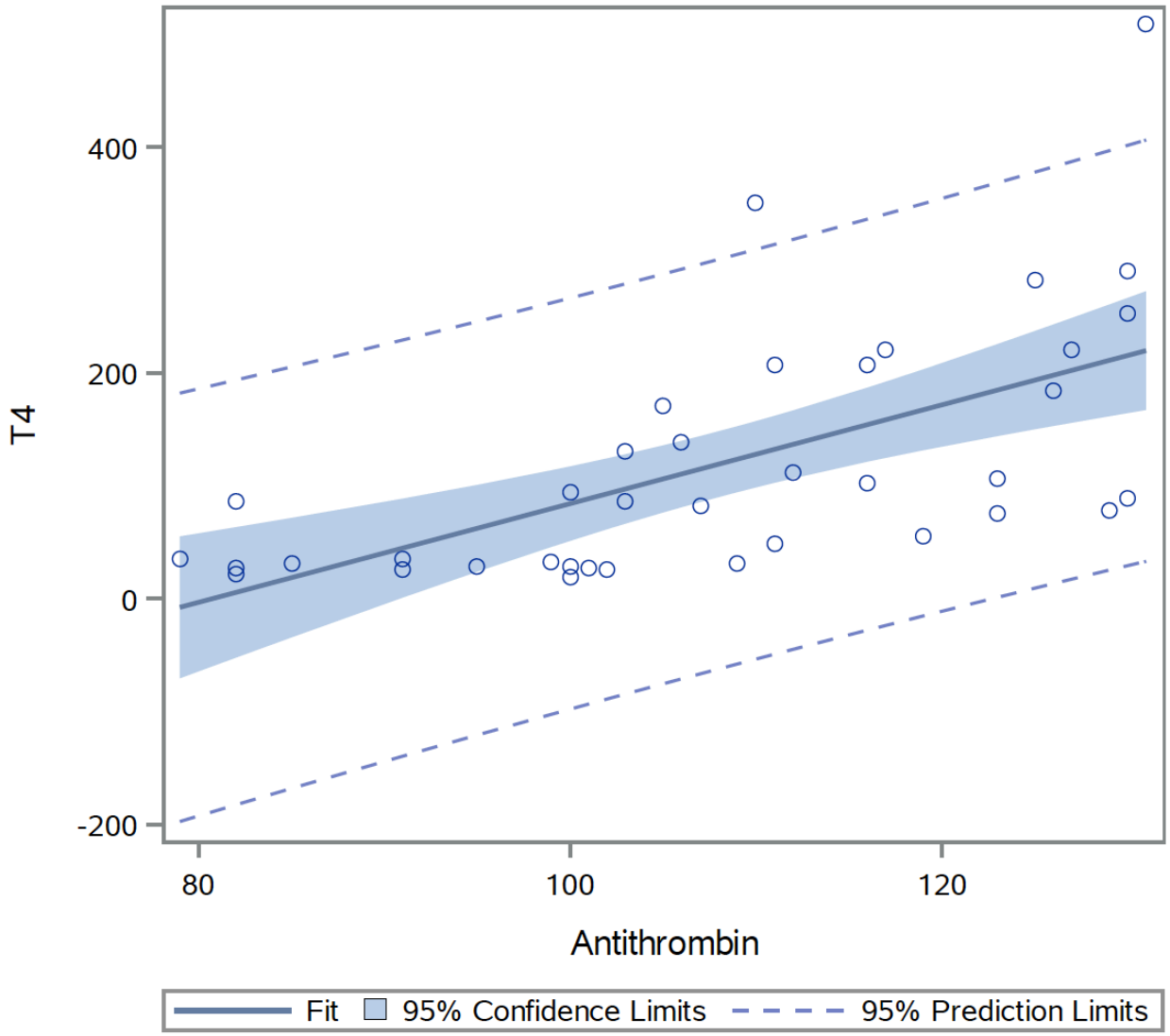
The median plasma D-dimer concentration was significantly increased post-RIT (n=7; median 389 ng/mL; range 98-890 ng/mL). P-value was obtained by Wilcoxon signed-rank test. A p-value <0.05 was considered significant.

Figure 8. Correlation between serum T4 and fibrinogen concentration



Linear regression analysis showing strong positive correlation between serum T4 value (nmol/L) and plasma fibrinogen (mg/dL) (r = 0.79; 95% CI 0.63 - 0.89) in hyperthyroid and control cats (n=38).

Figure 9. Correlation between serum T4 and antithrombin activity



Linear regression analysis showing strong positive correlation between serum T4 value (nmol/L) and antithrombin (%) (r = 0.70; 95% CI 0.50 - 0.84) in hyperthyroid and control cats (n=38).

APPENDIX B: TABLES

Table 1. Markers of hemostasis in hyperthyroid cats with normal and abnormal echocardiograms

Marker of Hemostasis	Normal Echo (n=6)	Abnormal Echo (n=19)	Reference Intervals and Units	P-value
PT	17.8 (16.9-18.7)	17.4 (16.0-18.7)	15.0-20.0 seconds	0.2743
aPTT	14.9 (12.0-16.7)	15.8 (12.0-30.4)	15.0-21.0 seconds	0.4336
Fibrinogen	235.0 (171.0-303.0)	337.0 (185.0-490.0)	76-270 mg/dL	**0.0253**
AT	119.0 (100.0-130.0)	116.0 (82.0-131.0)	75-110 %	0.9497
D-dimer	342.0 (0.0-1411.0)	139.0 (0.0-676.0)	0-250 ng/mL	0.3809
vWF:Ag	155.0 (132.0-221.0)	180.0 (112.0-253.0)	70-180 %	0.1939
TAT	12.8 (3.8-124.0)	5.7 (2.6-46.1)	1-8 ug/L	0.1651
Factor VIII	87.0 (41.0-495.0)	92.0 (18.0-388.0)	50-200 %	0.6672
Factor IX	114.0 (67.0-277.0)	110.0 (45.0-176.0)	50-150 %	0.7961

Data presented as median (range) with p-value obtained by Mann-Whitney U test. A p-value <0.05 was considered significant.

Table 2. Individual markers of hemostasis that supporting a hypercoagulable state

Markers of Hemostasis	Hyperthyroid cats	Control cats
aPTT	36% (9/25)	38% (5/13)
PT	0% (0/25)	0% (0/13)
Fibrinogen	56% (14/25)	0% (0/13)
Antithrombin	0% (0/25)	0% (0/13)
D-dimer	32% (8/25)	23% (3/13)
vWF:Ag	40% (10/25)	15% (2/13)
TAT	40% (10/25)	30% (4/13)
Factor VIII	20% (4/20)	23% (3/13)
Factor IX	30% (6/20)	30% (4/13)

Data presented as prevalence of the individual markers of hemostasis in both hyperthyroid cats and control cats that are outside of the reference interval in direction of hypercoagulability.

Table 3. Number of hemostasis markers that support hypercoagulable state in hyperthyroid and normal cats

	Hyperthyroid cats (n=25)	Control cats (n=13)
Mean	2.26086957	1.85714286
Range	0-6	0-5
Median	2	1

Data presented as the number of individual markers of hemostasis in both hyperthyroid cats and control cats that are outside of the reference interval in direction of hypercoagulability.

Table 4. Markers of hemostasis in hyperthyroid and euthyroid control cats

Marker of Hemostasis	Hyperthyroid	Control	Reference Intervals and Units	P-value
PT	17.4 (16.0-18.7)	17.1 (16.0-19.0)	15.0-20.0 seconds	0.8527
aPTT	15.5 (12.0-30.4)	15.0 (13.0-19.0)	15.0-21.0 seconds	0.7136
Fibrinogen	303.0 (171.0-490.0)	164.0 (123.0-218.0)	76-270 mg/dL	**0.0001**
AT	116.0 (82.0-131.0)	95.0 (79.0-109.0)	75-110 %	**0.0001**
D-dimer	139.0 (0.0-1411.0)	131.0 (48.0-1226.0)	0-250 ng/mL	0.8425
vWF:Ag	175.0 (112.0-253.0)	144.0 (89.0-214.0)	70-180 %	**0.011**
TAT	6.2 (2.6-124.0)	4.8 (2.6-44.4)	1-8 ug/L	0.4556
Factor VIII	89.5 (18.0-495.0)	97.0 (37.0-369.0)	50-200 %	0.9563
Factor IX	112.5 (45.0-277.0)	107.0 (50.0-285.0)	50-150 %	0.6351

Data presented as median (range) with p-value obtained by Mann-Whitney U test. A p-value <0.05 was considered significant.

Table 5. Markers of hemostasis in initial hyperthyroid and euthyroid cats post RIT

Marker of Hemostasis	Initial Hyperthyroid	Recheck Euthyroid	Reference Intervals and Units	P-value
PT	17.0 (16.7-18.3)	17.7 (17.0-18.6)	15.0-20.0 seconds	0.1563
aPTT	16.1 (13.5-18.0)	16.0 (14.5-18.3)	15.0-21.0 seconds	0.8125
Fibrinogen	337.0 (210.0-448.0)	178.0 (142.0-277.0)	76-270 mg/dL	**0.0156**
AT	123.0 (103.0-130.0)	112.0 (85.0-122.0)	75-110 %	**0.0156**
D-dimer	138.0 (0.0-683.0)	389.0 (98.0-890.0)	0-250 ng/mL	**0.0156**
vWF:Ag	211.0 (180.0-247.0)	175.0 (125.0-217.0)	70-180 %	**0.0156**
TAT	10.5 (2.6-46.1)	11.5 (2.0-16.1)	1-8 ug/L	1.0000
Factor VIII	84.0 (18.0-144.0)	109.5 (59.0-174.0)	50-200 %	0.0625
Factor IX	106.0 (45.0-154.0)	111.0 (74.0-175.0)	50-150 %	0.4375

Data presented as median (range) with p-value obtained by Wilcoxon signed-rank test. A p-value <0.05 was considered significant.

Table 6. Markers of hemostasis in sedated and unsedated cats (both hyperthyroid and control)

Marker of Hemostasis	Sedated	Unsedated	Reference Intervals and Units	P-value
PT	17.1 (16.0-18.7)	17.8 (16.9-19.0)	15.0-20.0 seconds	0.1354
aPTT	15.0 (12.0-30.4)	15.7 (13.0-20.4)	15.0-21.0 seconds	0.3594
Fibrinogen	256.5 (123.0-490.0)	214.0 (134.0-337.0)	76-270 mg/dL	0.2655
AT	108.0 (82.0-131.0)	106.5 (79.0-130.0)	75-110 %	0.9875
D-dimer	140.5 (0.0-1411.0)	114.5 (0.0-683.0)	0-250 ng/mL	0.4555
vWF:Ag	157.5 (100.0-253.0)	160.5 (89.0-221.0)	70-180 %	0.8151
TAT	7.4 (2.6-124.0)	4.5 (2.6-44.4)	1-8 ug/L	0.185
Factor VIII	97.0 (18.0-495.0)	82.0 (37.0-388.0)	50-200 %	0.4059
Factor IX	114.0 (45.0-277.0)	105.0 (50.0-285.0)	50-150 %	0.643

Data presented as median (range) with p-value obtained by Mann-Whitney U test. A p-value <0.05 was considered significant.