Effect of Levothyroxine Administration on Hemostatic Analytes in Doberman Pinschers with von Willebrand's Disease

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EFFECT OF LEVOTHYROXINE ADMINISTRATION ON HEMOSTATIC ANALYTES IN DOBERMAN PINSCHERS WITH VON WILLEBRAND'S DISEASE

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ABSTRACT

This study tested the hypothesis that levothyroxine supplementation increases plasma von Willebrand factor (vWf) concentration and enhances vWf function. The effects of levothyroxine administration were evaluated in 8 euthyroid Doberman Pinschers with plasma vWf concentration <30%. Levothyroxine (0.04mg/kg PO q12hours) and placebo were administered for 30 days in a 2-period, 2-treatment, double-blinded, crossover design with a 30-day washout period between treatments. Buccal mucosal bleeding time (BMBT), vWf antigen concentration (vWf:Ag), vWf collagen binding activity (vWf:CBA), Factor VIII coagulant activity (FVIII:C), serum total thyroxine (T4), free thyroxine (fT4), 3,5,3'-triiodothyronine (T3), and thyroid stimulating hormone were measured on days 0, 2, and 30 of each treatment period.

The dogs had markedly low plasma vWf:Ag concentrations (mean 8.9%; reference range 70-180%) and vWf:CBA (mean 11.1%; reference range >70%). All dogs had FVIII:C activity within reference range. The response to placebo versus active levothyroxine treatment revealed no significant differences between groups at any time for BMBT, vWf:Ag, vWf:CBA, and FVIII:C. Serum total thyroxine, fT4, and T3 were significantly higher in the levothyroxine-treated group compared to the placebo group at days 2 and 30. Thyroid stimulating hormone was significantly lower in the levothyroxine-treated group compared to the placebo group at days 2 and 30. Levothyroxine (0.04mg/kg) caused laboratory evidence of hyperthyroidism but did not affect plasma FVIII:C and vWf:Ag concentration or the vWf-dependent functional parameters of collagen binding and BMBT. The results of this study do not reveal a direct action of levothyroxine supplementation on plasma vWf concentration or activity in euthyroid Doberman Pinschers.

DEDICATION

This thesis is dedicated in memory of Dr. Bernard Feldman.

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LIST OF ABBREVIATIONS

ALT	Alanine transferase
AVP	Arginine vasopressin
BMBT	Buccal mucosal bleeding time
BT	Bleeding time
cAMP	Cyclic adenosine monophosphate
CBC	Complete blood cell count
CP	Cryoprecipitate
CU/dl	Canine units per deciliter
DDAVP	1-desamino-8-D-arginine vasopressin
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
EIA	Electroimmunoassay
ELISA	Enzyme-linked immunosorbent assay
FFP	Fresh frozen plasma
FP	Fresh plasma
fT4	Free thyroxine
FVIII:C	Coagulation Factor VIII
GP	Glycoprotein
IFN-γ	Interferon-γ
IL	Interleukin
mRNA	Messenger ribonucleic acid
OPD	O-phenylenediamine
PAI-1	Plasminogen activator inhibitor type 1
PR	Platelet glass bead retention
OSPT	One stage prothrombin time
aPTT	Activated partial thromboplastin time
rvWf	Recombinant von Willebrand factor
SDS	Sodium dodocyl sulphate

T3	3,5,3’-triiodothyronine
T4	Total thyroxine
TNF-α	Tumor necrosis factor-α
t-PA	Tissue-plasminogen activator
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
vWd	von Willebrand’s disease
vWf	von Willebrand factor
vWf:Ag	von Willebrand factor antigen
vWf:CBA	von Willebrand factor collagen binding activity

INTRODUCTION

Hemostasis involves the interaction of blood vessels, platelets, and the coagulation cascade. Following small vessel trauma, primary hemostasis is initiated as platelets adhere to the endothelium, where they encounter collagen, which activates platelets to secrete agonists. These agonists activate additional platelets flowing into the area, enabling them to adhere to platelets already on the endothelium. Through aggregation, a platelet plug forms occluding the injured vessel.[1] Primary hemostasis is then followed by secondary hemostasis, in which fibrin stabilization of the platelet plug occurs.[1] Von Willebrand factor (vWf) plays a critical role in the adherence of platelets to the vessel wall at the site of vascular injury, and in the absence of vWf, primary hemostasis is abnormal.[1,2]

Qualitative or quantitative deficiencies of vWf result in the bleeding disorder known as von Willebrand's disease (vWd).[1,3,4] Von Willebrand's disease is the most common congenital bleeding disorder of dogs and humans, but is reported in other species including the cat, rabbit, pig, horse, and cow. [5,6] This disease is recognized in over 50 dog breeds, as well as in mixed breed dogs.[5] Von Willebrand's disease can range from a subclinical disorder to a severe bleeding diathesis.[5,7] Treatment options for this disease are limited, and those available increase plasma vWf levels only transiently. Desmopressin (1-desamino-8-D-arginine vasopressin or DDAVP) can be administered, and is believed to release intracellular stores of vWf, but there is marked variation in individual dog response to this drug.[8] Alternatively, vWf can be administered in plasma transfusions, although repeated use of blood products is associated with an increased risk of adverse immunological reactions or volume overload.[5] Cryoprecipitate (CP) contains vWf in a smaller volume but availability of this product limits its use.[1,5] Administration of oral levothyroxine has been described as a treatment option for canine vWd. Elevations in vWf concentrations may occur within 48 hours of initiating therapy, but these results have not been completely substantiated.[9] The objective of this study was to test the hypothesis that levothyroxine supplementation acts to increase plasma vWf

concentration and enhance vWf function when administered to euthyroid Doberman Pinschers with type I vWd.

CHAPTER I: LITERATURE REVIEW

A. Von Willebrand factor structure and function

Von Willebrand factor is a multimeric, adhesive glycoprotein composed of polypeptide subunits linked by disulfide bonds and is the largest plasma protein.[1,6,7] Multimers of vWf have variable weights, due to differences in the number of subunits from which they are comprised.[10] Von Willebrand factor plays a crucial role in primary hemostasis through the following sequence of events. First, vWf binds to subendothelial components and then to platelet receptor glycoprotein (GP) Ibα, leading to initial platelet adhesion. A second platelet membrane receptor, $\alpha_{IIb}\beta_3$, is expressed, and binding of vWf to $\alpha_{IIb}\beta_3$ leads to irreversible platelet adhesion and platelet aggregation.[11]

In the intact blood vessel, vWf does not bind platelet receptors.[10,12] It is assumed that in the injured blood vessel, exposed subendothelial structures bind vWf, which induces a conformational change in vWf, leading to exposure of binding sites for platelet adhesion.[10] It is not known which subendothelial ligands are physiologically important for binding vWf , but vWf binds to fibrillar collagen types I, III, and VI of the vessel wall and to noncollagenous components of the subendothelium.[10,11] Each multimer of the vWf molecule contains discrete binding sites for collagen and platelet receptors GPIbα and $\alpha_{IIb}\beta_3$.[7,10]

Following subendothelial binding, vWf binds GPIbα on platelet membranes, which serves to provide the major adhesive link between the platelet and vessel wall at the site of vascular injury. This initial link between vWf and GPIbα results in reversible platelet binding called platelet adhesion.[11] Binding of vWf to GPIbα induces platelet activation, resulting in expression of the complex $\alpha_{IIb}\beta_3$ on the platelet membrane.[10] Activated $\alpha_{IIb}\beta_3$ cross-links adjoining platelets via bridges made by vWf, fibronectin, vitronectin, and other proteins.[10] This process is called platelet aggregation and results in irreversible adhesion of platelets to the subendothelium. The binding of vWf to $\alpha_{IIb}\beta_3$ is considered a

requirement for firm attachment of platelets that can resist the high shear forces in circulating blood.[11] This process operates at both arterial and venous levels of shear.[13]

Von Willebrand factor also serves as a carrier molecule for coagulation factor VIII (FVIII:C) as they circulate noncovalently bound.[1,7] This interaction serves to protect FVIII:C from inactivation by activated protein C and factor Xa in circulation.[10,11,14] Additionally, vWf may assist in localizing FVIII:C to the site of platelet plug formation, where it serves as cofactor in the generation of factor Xa.[11] In humans with deficient quantities of vWf, FVIII:C deficiency results.[11] In dogs with vWd, FVIII:C deficiency is not pronounced, because canine plasma normally contains 2 to 3 times more FVIII:C than human plasma.[15]

Synthesis of vWf occurs in the vascular endothelial cells and megakaryocytes.[3,14,16] In endothelial cells, vWf is stored in specialized organelles called Weibel Palade bodies and is secreted into the plasma constitutively or deposited as a component of the subendothelial matrix bound to collagen.[14] In contrast, megakaryocyte vWf is packaged in platelet α granules.[14] In cultures of endothelial cells, predominantly small multimers are secreted constitutively, whereas large multimers are rapidly released by factors that cause endothelial activation.[17] Canine platelets contain little vWf.[7,18] Platelet α granules contain 10-37% of total body vWf concentration in humans and only 2-3% in the dog.[6] Most human studies have found that high molecular weight multimers predominate in platelets.[18] Parker et al detected ultra-high-molecular-weight vWf multimers in platelet lysates of 2 of 5 normal dogs studied.[18] The physiologic mechanisms that control the release of vWf and regulate normal plasma levels are poorly understood.[3] In vitro, vWf release from platelets is stimulated by exposure to collagen, adenosine diphosphate, or thrombin, and the release of vWf from endothelial cells is mediated by thrombin, fibrin, and histamine.[3,17] A theory of neuroendocrine regulation has been proposed based on observations that manipulation of the fourth ventricle and administration of vasopressin or its analogue desmopressin, result in increased levels of vWf in normal humans.[3] Similarly, increases in vWf occur in normal dogs administered desmopressin.[19]

Von Willebrand factor is synthesized as a precursor form termed pre-pro-vWf. [14,16] Its dimers are assembled in the endoplasmic reticulum and in the Golgi apparatus, where dimers form multimers.[14,16] After post-translational modifications, endothelial cells secrete vWf or store it in secretory vesicles called Weibel Palade bodies, in the form of pro-vWf.[6,16,17] Here it is cleaved to mature vWf and pro-polypeptide.[6] Proteolytic enzymes regulate the polymeric size of vWf.[10] The largest multimers are present in the endothelial cells, subendothelium, and platelets and are only transiently observed in normal blood.[10,11] These large multimers are the most hemostatically active, with increased capability for binding collagen and platelets, but not FVIII:C.[20]

B. Canine von Willebrand's disease

1. Classification of canine von Willebrand's disease

In the dog, vWd is classified as 1 of 3 types based on the plasma concentration of vWf and the relative number of high molecular weight multimers present. Type I vWd is characterized by subnormal plasma concentrations of vWf, having the same array of multimeric sizes as found in normal plasma.[5] This is the most common form of the disease in humans and canines and affects many dog breeds, including Doberman Pinschers.[5,6] In type I vWd the plasma deficit of vWf results from decreased endothelial cell production of vWf .[6,21] Type I vWd is inherited as an autosomal dominant trait with incomplete or variable penetrance.[6]

Dogs with type II vWd lack larger vWf multimers.[5] Breeds affected with type II vWd include the German shorthaired pointer and German wirehaired pointer in which it is inherited as an autosomal recessive disorder.[6,22,23]

Type III vWd is the most severe form and is characterized by a lack or trace amount of all multimers of vWf protein.[5] Type III vWd is reported in Scottish Terriers, Chesapeake Bay Retrievers and Shetland sheepdogs, as well as other breeds.[5,6] Type III disease is inherited as an autosomal recessive trait with homozygotes having extremely low or undetectable vWf:Ag concentration.[6]

2. Incidence and inheritance of von Willebrand's disease in Doberman Pinschers

It is difficult to assess the incidence of vWd in dogs because hemorrhagic episodes may go unnoticed.[6] Von Willebrand's disease has been identified in over 50 dog breeds, with a particularly high prevalence in certain breeds.[5] Up to 70% of Doberman Pinschers have Type I disease with vWf concentrations below 50%.[24] Despite the high prevalence in this breed, bleeding episodes are relatively uncommon.[7] In Doberman Pinschers vWd is inherited as an autosomal trait with incomplete or variable penetrance.[5] As the trait is autosomal, males and females have an equal incidence of transmitting the defect or expressing a bleeding diathesis.[5,24,25] The mutation causing type I vWd in Doberman Pinschers appears to be a splice site mutation, with alternate splicing occurring 90-95% of the time.[6] Inheritance of vWd in this breed may result from a single gene defect in which each normal allele produces half the normal amount of vWf produced when both alleles are normal. Each defective allele results in a phenotype producing a vWf:Ag concentration of less than 15 CU/dL. Thus dogs with low levels would be homozygous for the defective allele and dogs with midrange concentrations would be heterozygous for the defect.[26] In contrast to early opinion, homozygosity is not considered to be lethal.[27]

3. Clinical signs of canine von Willebrand's disease

The clinical presentation of vWd-affected dogs ranges from subclinical to a severe bleeding diathesis.[5,7] The severity of bleeding depends on factors other than vWf, such as anatomic site, type of insult, amount of tissue injured, and ease of control of hemostasis at the given location.[5] In general, signs of type I disease are mild to moderately severe, signs of Type II are often severe, and signs of type III are often the most severe.[6] In one study, greater than 90% of bleeding episodes in affected dogs required some form of intervention to control the hemorrhage.[24] Bleeding episodes may be precipitated by physical, emotional, or physiologic stresses, including concurrent diseases, or by concurrent disorders that affect hemostasis.[5,9,28] Type I vWd-affected dogs with vWf concentrations <36% are considered at increased risk of hemorrhage, although some dogs with values above that cutoff also exhibit bleeding.[25] Johnson et al found that Doberman Pinschers with vWf concentrations of ≤30% tended to hemorrhage more during

otoplasty.[29] In many cases, clinical signs first occur in adult dogs, and the mean age of diagnosis in Doberman Pinschers in one study was 4 years of age.[5,29] In human patients, the variable presentation of bleeding symptoms in patients with vWd remains poorly understood.[10]

The severity of vWd-related bleeding ranges from mild to severe, and may even be fatal.[25,28] Bleeding may be traumatically induced or non-induced.[24] Manifestations of mucosal bleeding include prolonged estrual and post-partum hemorrhage, urethral hemorrhage, hematuria, melena, hematochezia, gingival hemorrhage, and epistaxis. Nonmucosal hemorrhage includes prolonged bleeding after trauma (surgical trauma or wounds), excessive bleeding from nail cutting, subcutaneous hematomas, hemothorax, hemarthrosis, hyphema, neonatal umbilical bleeding and subarachnoid space hemorrhage. In addition, serosanguineous otitis externa, abortions, stillbirths, and neonatal deaths have been described with canine vWd .[7,22] In one study of Doberman Pinschers, 47% of mucosal bleeding was from the urogenital tract.[24]

4. Diagnostic testing for von Willebrand's disease

Von Willebrand's disease results from a quantitative or qualitative abnormality in vWf. [1,3,4] Because the larger multimers are more hemostatically active, a relative deficiency of large multimers may decrease vWf activity.[20] Tests to qualitatively evaluate vWf include evaluation of bleeding time, platelet glass bead retention, platelet agglutination studies, automated platelet function analysis, and collagen binding assays. Plasma concentrations of vWf may be evaluated by antigenic determinations, and multimeric analysis may be performed to determine the distribution of the various sized multimers. Deoxyribonucleic acid (DNA) testing may further assist in evaluation of dogs used for breeding purposes.

Bleeding time (BT) is considered the most reliable indication of *in vivo* primary hemostatic capabilities.[7,30,31] Bleeding time is considered a useful screening test for vWd, but it is not a specific test for vWd, as it is affected by abnormalities or deficiencies of vWf, platelets, and vascular integrity.[5,30,32] There are several methods by which BT may be evaluated. To evaluate direct microscopic bleeding time in the dog, a subcuticular

vessel of the medial thigh or a mesenteric vessel is isolated and transected and the BT is determined by microscopic examination.[30] Dermal BT is evaluated in the anesthetized dog using an incision made on the skin of the thigh, but results are highly variable depending on skin thickness, age, breed, and amount of subcutaneous fat.[30,32] In humans the dermal BT is performed on the inner arm and is the preferred technique. Also in the anesthetized dog, a block resection of gingiva may be performed to evaluate the template gingival BT or the apex of the cuticle may be severed to measure cuticle BT.[30] The cuticle BT is difficult to standardize and does not differentiate between abnormalities of primary and secondary hemostasis.[32] The buccal mucosal BT (BMBT) consists of making a small, shallow incision with a commercial template device in the buccal mucosa of the dog to produce a consistent incision.[32] This procedure is well-tolerated, fairly non-invasive, does not require anesthesia, requires minimal equipment, and is easy to perform.[30,33]

The BMBT is considered a sensitive test of primary hemostatic capabilities and may be the best way to assess the clinical expression of vWd.[6,33] Abnormalities of BMBT are not specific for vWd and may also occur as a result of abnormal platelet number or function, abnormal vascular integrity, metabolic disorders such as uremia, and following the administration of drugs including aspirin, ibuprofen, dextrans, carbenicillin or ticarcillin.[32,34] Normal canine BMBT is approximately 2-4 minutes, regardless of gender.[32,34] Whether bleeding time correlates with risk of hemorrhage is controversial.[35] Jergens et al reported that the BMBT for dogs that had normal hemostasis was significantly shorter than the BMBT for the dogs that had deficient hemostasis during surgical procedures.[34] Doberman Pinchers with vWf concentrations <30% are likely to have prolonged BMBTs, although the BMBT may not correlate directly with plasma vWf concentrations.[34,36]

Platelet glass bead retention (PR) is a general assay of hemostasis in which blood is passed over a plastic column packed with glass beads. The platelet concentration is measured before and after a known volume of blood has been passed over the column and the percentage of platelet retention is calculated. Normal PR is >70% and dogs

affected with vWd have PR values between 40-66%.[21] Platelet retention is useful as a screening test, but is neither specific nor accurate, as PR values are dependent not only on the presence of vWf, but also on normal red blood cell counts and a functional coagulation cascade.[6,30] Disorders causing platelet dysfunction, such as renal failure and aspirin administration, also cause abnormalities of PR.[6,30] In vWd-affected dogs treated with cryoprecipitate, BTs have been found to decrease without concurrent alterations in PR.[15]

Platelet agglutination reactions are based on the ability of certain agents to bind to each other in a vWf-dependent manner. To perform these assays, fixed platelets adhered on a surface are mixed with patient plasma. An agglutination agent, such as ristocetin or botrocetin, is added and the rate of agglutination, which is proportional to the activity of vWf in the plasma, is measured.[12] Agglutination can be determined by visual assessment, making consistency in determination of the endpoint difficult.[12] Alternatively, agglutination can be measured using a platelet aggregometer to generate a curve where the slope of the curve indicates the rate of agglutination. The agglutination rate of the patient plasma is compared to serial dilutions of normal pooled plasma and the results are reported as % activity in which the normal pooled activity is considered to be 100%.[12] Unfortunately, standardization of platelet agglutination results is difficult.[12]

Botrocetin, a protein from the venom of certain pit vipers, binds vWf near the GPIbα binding site, promoting vWf-mediated platelet agglutination.[10] Measured this way vWf is called botrocetin cofactor. The measured result is semiquantitative, reflecting both vWf concentration and function.[5] This assay is considered imprecise and difficult to standardize.[5]

Similarly, the ristocetin assay reflects vWf concentration and function. The platelet-agglutinating property of vWf evaluated by this assay is termed ristocetin cofactor activity.[10] The interaction of vWf with the positively charged antibiotic ristocetin, results in exposure of the GPIbα binding site and the resulting platelet agglutination by vWf in plasma is determined turbidimetrically.[10,11] This test is technically difficult to perform using canine platelets and is considered to be insensitive.[5,37]

A recently developed automated platelet function analyzer[a] identifies dogs with vWd and other platelet abnormalities.[38,39] Blood is passed through a capillary device to mimic *in vivo* high shear conditions. The end-point is determined when flow through the instrument ceases as a result of platelet adhesion and subsequent aggregation, following exposure to platelet agonists coated onto a membrane in a disposable cartridge device. This test is very sensitive to vWf abnormalities and may be a superior screening tool, although results are not specific for vWd.[39,40] While this analyzer provides a convenient point-of-care assay, it remains to be determined whether this test can distinguish various subnormal levels of vWf in dogs (ie., severe deficiencies from mild deficiencies) and whether it provides a good prediction of the risk of surgical bleeding.[39]

The collagen binding assay is an evaluation of vWf's functional activity, based on the protein's ability to bind canine collagen *in vitro*.[37] Von Willebrand factor collagen binding activity (vWf:CBA) is quantified using an enzyme-linked immunosorbent assay (ELISA) technique. Fresh canine tendon is used as the collagen source to generate a collagen solution that is then coated and incubated on a microtiter plate. Sample plasma is added and following incubation and washing, a peroxidase-conjugated rabbit anti-human vWf antibody is added. After incubation, O-phenylenediamine (OPD) is applied and the OPD solution is activated by the addition of hydrogen peroxide. This reaction is stopped by the addition of sulfuric acid and the optical densities of the wells are read using a microplate reader. Plasma vWf:CBA of the test sample is measured against a normal pooled canine plasma designated as 100%.[37] In normal dogs there is a close correlation between the plasma concentration of vWf and vWf:CBA.[37] Decreased vWf:CBA has been demonstrated in plasma with reduced quantities of high molecular weight multimers and thus may reflect the *in vivo* activity of vWf.[37] This test is more sensitive and easier to perform than the ristocetin and botrocetin agglutination assays.[37]

Plasma concentrations, regardless of biologic activity, may be quantitated as vWf antigen (vWf:Ag) concentration.[6,7] Antibodies directed against vWf protein antigens are used to determine the concentration of vWf in a sample, which is reported as a percentage compared to standard control plasma or as canine units/deciliter (CU/dl).[5] Older

techniques employed the "Laurell rocket" electroimmunoassay (EIA) whereas newer techniques use an ELISA assay.[6] Compared to the EIA, the ELISA technique is more sensitive, more accurate, easier to perform, and provides reliable same-day results.[6,12] As well, the ELISA is better suited to testing a large number of samples.[12] Definitive diagnosis of vWd requires specific determination of plasma vWf with a result lower than 49% compared to a reference pool considered to be 100%.[6] Dogs with vWf:Ag concentrations between 70-180% are normal, between 50-69% are borderline, and between 0-49% are abnormal.[5]

An individual's vWf:Ag concentration has been used to predict genotype. Dogs with values in the normal range are considered not to have the vWd trait and are considered to be at low risk for expressing or transmitting the disease. Some dogs with vWf:Ag concentrations between 50-69% may be genetic carriers of vWd. Dogs with vWf:Ag concentrations <70% are considered carriers of vWf and are at risk for transmitting an abnormal vWf gene to their offspring.[5] Most Doberman Pinschers with Type I vWd that are homozygous "affected" have vWf concentrations < 50%. However, the severity of bleeding in an individual dog is not necessarily proportionate to their plasma concentration of vWf:Ag.[5]

Samples that are clotted or severely hemolyzed are invalid for analysis.[5] Poor sample handling may degrade the sample, resulting in overestimation of vWf:Ag concentration, or destroy the antigenic sites, resulting in underestimation.[7] The most reliable results are obtained from samples drawn during physiologic "quiet" times, as vWf is an acute-phase reactant protein and fluctuates unpredictably during illness and other physiologic states.[5]

Electrophoresis or multimeric analysis may be used to estimate the relative abundance of the different sizes of vWf multimers, providing a qualitative assessment of vWf.[6] Agarose gel electrophoresis is most commonly used, as it is capable of a much higher resolution than the older crossed immunoelectrophoresis method.[7] When a plasma sample is electrophoresed in sodium dodocyl sulphate (SDS)-agarose gel, the lower molecular weight multimers stay closer to the origin, such that the different sizes of vWf multimers are separated into bands. The multimeric bands can be visualized using anti-

vWf antibodies bound to ^{125}I allowing visualization of the bands by autoradiography. Alternatively the bands may be visualized using horseradish peroxidase-conjugated antibody after electroblotting. The pattern of the bands from the test sample can then be compared to the pattern of bands obtained from pooled canine plasma from normal individuals.[7,12] Multimeric analysis is technically complex and time consuming and is impractical for large volume screening; however it allows categorization of vWd according to the presence or absence of various sized multimers. [5,37]

For some breeds, including Doberman Pinschers, DNA testing is available that allows identification of dogs as affected (homozygous positive), carriers (heterozygous), or unaffected (homozygous negative) for vWd.[22] This information may be used to make decisions about breeding selection. Oral epithelial cells are collected with a small brush and submitted.

Von Willebrand factor circulates noncovalently bound to FVIII:C and serves to protect FVIII:C from inactivation.[10,11,14] In dogs with vWd, FVIII:C levels seldom drop below 20%, so activated partial thromboplastin time (aPTT) is typically normal or only slightly prolonged.[7] However, decreases in FVIII:C may be more pronounced in dogs with marked decreases in vWf concentrations.[41]

5. Management of canine von Willebrand's disease

The management of canine vWd includes avoidance of hemorrhage and treatment of bleeding episodes. For dogs with severe forms of vWd, invasive diagnostic procedures and elective surgical procedures should be avoided when possible.[5] Careful hemostatic techniques and close intra-operative and post-operative monitoring are advisable, since delayed onset of bleeding has been reported as long as 24 hours after surgery in dogs with vWd.[5] Following administration of vaccines that cause transient thrombocytopenia, surgery should be postponed until normal platelet numbers are present.[5,9] Similarly, drugs that impair platelet function or production should be avoided.

Desmopressin (1-desamino-8-D-arginine vasopressin or DDAVP) is a synthetic analogue of vasopressin that increases plasma vWf concentrations, likely through stimulating the release of intracellular vWf stores. [3,5] There are 2 types of vasopressin

receptors in the body: V1 receptors regulate phophatidylinositol-dependent vasoconstriction and V2 receptors are involved in a cyclic adenosine monophosphate (cAMP)-dependent antidiuretic action as well as the release of FVIII:C and vWf .[3,42] Desmopressin has minimal smooth muscle activity and acts mainly as a V2 receptor agonist, inducing vWf secretion from Weibel Palade bodies by exocytosis.[3,42] DDAVP is thought to stimulate the release of pre-formed vWf stored in endothelial cells because of the rapidity of the response and the appearance of high molecular weight multimers typical of the multimers released from Weibel Palade bodies.[42] In addition to elevations in vWf concentrations, DDAVP also results in increases in FVIII:C and tissue-plasminogen activator (t-PA).[42]

Desmopressin may be administered as an intravenous infusion, subcutaneous injection, or intranasally.[43] Subcutaneous administration is as effective as intravenous administration in most cases, but intranasal administration has shown variable efficacy.[44] In humans, intravenous infusions have been shown to raise plasma vWf levels 3-5 times above the baseline level with elevations lasting 8-10 hours, and infusions can be repeated every 12-24 hours as needed.[43] Side effects of DDAVP are generally mild and include mild tachycardia, headache and a decrease in mean arterial pressure.[45] Serious side effects such as hyponatremia and volume overload are uncommon or rare.[43,45] DDAVP is much less effective in patients with type II or type III vWd.[16,43]

Administration of DDAVP does not consistently increase circulating vWf in dogs.[46,47] When given to normal dogs, DDAVP increases the concentration of plasma vWf only slightly.[46] Johnstone et al found that DDAVP induced increases in vWf:Ag concentration in normal dogs (vWf:Ag increased by 57%) and vWd-affected dogs (vWf:Ag increased by 60%) but there were disproportionately greater increases in vWf:CBA, suggesting that DDAVP selectively releases more functionally active vWf multimers.[48] This finding is supported by studies using multimeric analysis, which show an increase in the relative number of large multimers following treatment with DDAVP, along with an increase in botrocetin cofactor activity that exceeds the increase in vWf:Ag concentrations.[36,44]

Following intravenous administration of DDAVP to dogs, plasma vWf:Ag concentrations increase in about 10 minutes and remain elevated for more than 2 hours.[49] However, repeat administration results in a diminished response, suggesting that multiple dosing may not be effective.[49] In addition, marked variation in the individual response to DDAVP administration has been noted in dogs, particularly vWd-affected dogs.[8] This poor response to DDAVP may be the result of low levels of vWf in canine endothelial cells in dogs with vWd.[21] Although individual dog responses to DDAVP are variable, the response of an individual dog is highly reproducible; therefore, administration of a test dose has been advocated.[45,49] Furthermore, some experts recommend evaluating bleeding time following a test dose, prior to using DDAVP as a prophylaxis for surgery.[5] Shortening of BMBTs, which is disproportionate to the small increases in plasma vWf concentrations, has been reported in some Doberman Pinschers following treatment with DDAVP.[15,36] Alternatively, DDAVP may be administered to plasma donor dogs prior to collection, although the expected benefit to the recipient is not well described.[1,5,7]

Blood component transfusions are required to supply vWf to control hemorrhage in dogs with severe forms of the disease and dogs non-responsive to supportive care or DDAVP.[5] Von Willebrand factor may be administered as a component of cryoprecipitate (CP), fresh plasma (FP) or fresh frozen plasma (FFP) or as whole blood if concurrent administration of red blood cells is appropriate.[5] Blood donors should be screened to ensure adequate levels of vWf prior to inclusion in a donor program.[5] Fresh frozen plasma is prepared within 6 hours of collection and, if stored at –70^{o}C, may be preserved for as long as 1 year.[5] Transfusions of FP or FFP supply approximately the same amount of vWf in half the volume compared to whole blood.[5] Repeated use of whole blood products puts patients at risk for volume overload and adverse immunologic reactions.[5,43,50] A dose of 6-10 ml/kg body weight of FP or FFP has been suggested.[6] After FFP administration, vWf:Ag concentration increases to approximately 30 CU/dl, although a concurrent improvement in BMBT and PR is not seen.[15] It is possible that a better response would be achieved with a higher volume of FFP.[15]

Cryoprecipitate is considered to be the most effective and reliable means of controlling or preventing hemorrhage in dogs with vWd.[1,7] Cryoprecipitate is prepared by slowly thawing FFP at 4°C until a few ice crystals remain, then centrifuging the plasma to sediment the CP.[12] This precipitate is rich in high molecular weight vWf, fibrinogen, fibronectin and FVIII:C and can be used immediately or frozen for use as long as 6 months to 1 year later.[1,24,51,52] Cryoprecipitate contains 5-20 times the amount of vWf as FFP, allowing a large amount of hemostatically active vWf to be administered in a relatively small volume.[1,5,15,43] In Doberman Pinschers with type I vWd, administration of CP was shown to significantly increase vWf levels within 30 minutes of administration with the effect maintained for up to 4 hours. The effects of CP were also compared to those of FFP, and only CP shortened the BMBT despite an elevation in plasma concentration of vWf:Ag following FFP transfusion.[15] This difference was proposed to be the result of differences in the content of larger, more hemostatically active multimers or due to expanded blood volume after FFP but not CP administration.[15] The BMBT returned to baseline by 4 hours post-CP transfusion.[15] Stokol and Parry found CP to be effective at raising vWf:Ag concentrations above 35 CU/dl, but the change in BMBT did not correlate with the increase in vWf:Ag.[50] The recommended CP dose is 1 unit/10 kg of recipient body weight, but the definition of a unit ranges to include the amount of CP formed from 150 ml to 350 ml of plasma.[5,6,50] Cryoprecipitate is convenient with the entire dose being administered quickly pre-operatively and repeat doses can be given with reduced risk of volume overload.[5] Disadvantages of CP include reduced availability and increased technical ability and cost of production and storage.[22] Furthermore, transfusions only transiently elevate plasma vWf concentrations, limiting their practicality to peri-operative and emergency situations.

Additional concerns with the administration of blood products in human medicine include the inability to apply virucidal techniques to these products and the development of anti-vWf alloantibodies, which, after multiple transfusions, not only render the transfusions ineffective, but also may cause anaphylactic reactions.[43] Human recombinant vWf (rvWf) has been developed to overcome these limitations. Studies evaluating human

rvWf use have been performed in dogs with vWd and have demonstrated an immediate rise in ristocetin cofactor activity and vWf:Ag concentration, although the bleeding times did not shorten.[53] Cost, availability and the potential for formation of canine antibodies against the human rvWf are concerns facing veterinarians considering use of this product.

C. Relationship between canine von Willebrand factor and thyroid hormone

1. Acquired von Willebrand's disease and hypothyroidism

In human patients, acquired vWd has been reported in association with a wide variety of disorders including lymphoproliferative and myeloproliferative disorders, dysproteinemias, vascular disorders, and nephroblastomas.[3] Hypothyroidism is also associated with acquired vWd in humans. Thyroid replacement therapy in these patients results in elevation and normalization of plasma vWf:Ag concentrations.[54-61] Hypothyroidism-related acquired vWd is hypothesized to be the result of a circulating antibody that acts as a functional inhibitor or that promotes clearance of the circulating antibody complex by the reticuloendothelial system.[54,59,62-64] An alternative theory is that hypothyroidism results in an overall decrease in protein synthesis that affects the synthesis of vWf.[55,61]

Two case reports of possible canine acquired vWd are reported in the veterinary literature. The first is a report of a 4-year old male Doberman Pinscher with a presenting complaint of spontaneous epistaxis.[65] The dog was diagnosed with autoimmune anemia based on a positive Coomb's test, splenomegaly, and reduced red blood cell count. Von Willebrand's disease was diagnosed based on the vWf:Ag concentration which was undetectable (ie., <7%) and hypothyroidism was diagnosed based on low resting T3 and T4 values. Management included sedation, vitamin K1, packing of the nares, intravenous fluids, fresh whole blood transfusion, prednisone, amoxicillin, and levothyroxine. This dog was suggested to have acquired vWd secondary to hypothyroidism. However, the dog was not determined with certainty to be hypothyroid, but rather the low resting T3 and T4 values may have been due to euthyroid sick syndrome. Furthermore, vWf:Ag concentration was not re-evaluated following levothyroxine supplementation, so whether

the supplementation had any effect on the vWd remains undetermined. More likely, this dog had congenital vWd and the role of the concurrent diseases is questionable.

The second case report of possible canine acquired vWd describes a 14-month old Dachshund presented for bilateral swelling of the vulva and caudal mammary glands.[66] The dog was diagnosed with immune mediated thrombocytopenia (based on the low platelet count, bone marrow cytology, and positive platelet factor III assay) and was treated with prednisone. The FVIII:C and vWf:Ag concentrations were low and the dog was diagnosed with vWd. Serum T4 and TSH concentrations were decreased and normal respectively, and were attributed to corticosteroid therapy. Following DDAVP administration, ovariohysterectomy and splenectomy were performed and biopsies were taken of the swollen areas, which were consistent with lymphangioma. The authors of this report commented that the vWd was most likely congenital, rather than acquired. Some experts have discredited both of these reports and stated that acquired vWd has not been reported in dogs.[6,22]

2. Congenital von Willebrand's disease and thyroid hormone

As an alternative therapy for vWd in dogs, daily supplementation with thyroid hormone has been suggested to lessen or control mild to moderate bleeding and to increase plasma vWf:Ag concentrations.[9,28] Based on unpublished data, the successful treatment of 7 Doberman Pinschers with vWd and mild hypothyroidism is described. The dogs were given a standard manufacturer-recommended therapeutic dose of thyroxine, and a 2-3 fold increased in their baseline vWf activities occurred within 24 hours. The peak effect occurred at 3 days and was sustained for another 3-4 days.[9] Based on these results, Dodds recommends that all Doberman Pinschers presenting with serious bleeding have their thyroid function evaluated and patients be administered thyroid hormone pending test results.[9] The same author suggested that bleeding times in dogs with vWd and hypothyroidism were corrected from greater than 20 minutes to within the normal range, within 48 hours of initiating thyroxine supplementation. Dodds also recommends that both serum thyroid hormone concentrations and bleeding times be monitored to evaluate for a therapeutic response.[67] Prophylactic use of levothyroxine at 0.1 mg/4.5 kg

q 12 hours was advised beginning 48 hours prior to elective procedures, with continuation of the treatment beyond 10 days if the bleeding continues or if the patient has thyroid disease.[67] Thyroid hormone supplementation was hypothesized to promote hemostasis by improving platelet function, stimulating thrombopoeiesis, and enhancing release of vWf and other coagulation factors.[67] Unfortunately, the original data is unpublished, thus results remain unsubstantiated.

This report was followed by a study published by Avgeris et al in which the mean vWf:Ag concentration of 14 hypothyroid dogs was found to be significantly decreased compared with 14 euthyroid dogs.[68] Additionally, 4 of the hypothyroid dogs were supplemented with levothyroxine for 1 month and all had increased levels of vWf:Ag. This author concluded that thyroxine should be administered to hypothyroid dogs with low vWf concentrations.

A number of shortcomings are apparent when evaluating this study. Hypothyroidism was not well documented, as the dogs were diagnosed as hypothyroid based on thyrotropin releasing hormone (TRH) response tests, a method considered unreliable since only small increases in thyroxine levels normally follow the administration of TRH. Additionally, 10 of the 14 supposedly hypothyroid dogs had normal baseline thyroxine values, while other studies have shown that 89% of dogs with hypothyroidism have serum T4 concentrations below normal.[69] None of the 14 dogs had signs of hemorrhagic tendencies compatible with severe vWd. In addition, there was a breed bias, with 8 of the 14 dogs being Doberman Pinschers, a breed with a prevalence of vWf deficiency up to 70%. Only a small number of dogs were examined following supplementation. Two of these dogs had only very small increases in their vWf:Ag concentrations, so that *in vivo* or assay factors could account for the increases noted. This study suggested that hypothyroid dogs may have lower vWf:Ag concentrations than euthyroid dogs because certain dog breeds are predisposed to both diseases or because hypothyroidism may cause acquired vWd. This study also proposed that vWf:Ag concentrations will increase in dogs with hypothyroidism during levothyroxine administration. However, these hypotheses remained controversial.

To determine whether an association exists between vWd and hypothyroidism, Lumsden et al evaluated vWf:Ag concentrations, FVIII:C, and thyroid status based on T4 concentrations and TSH response tests in 75 Doberman Pinschers.[70] No association was found between hypothyroidism and vWd, indicating that Doberman Pinschers with hypothyroidism were no more likely to have low vWf:Ag concentrations than euthyroid Doberman Pinschers. This contradicted Dodds' unpublished study of 200 Doberman Pinchers, in which 61% had laboratory evidence of both vWd and hypothyroidism.[28] The study by Lumsden et al fails to support Dodds' claim that the concomitant occurrence of vWd and hypothyroidism in many dog breeds indicates a link between the synthesis or metabolic regulation of thyroid hormone and vWf.[28]

Johnstone et al, in an abstract, reported administering levothyroxine to 20 Doberman Pinschers and comparing their vWf:Ag concentrations at 6 and 12 weeks to 21 control dogs.[71] Their results showed that levothyroxine administration had no significant effect on vWf:Ag concentration or FVIII:C activity, irrespective of thyroid status. Unfortunately, these results were not published and therefore the design, methodology, and results cannot be critically reviewed.

Panciera et al measured plasma vWf:Ag concentrations in 10 dogs with spontaneous hypothyroidism before and after administration of levothyroxine for 67 +/- 12 days.[72] The vWf:Ag concentration was within normal reference range prior to supplementation and decreased following levothyroxine administration. These authors concluded that levothyroxine should not be administered to hypothyroid dogs with the expectation of elevating plasma vWf:Ag concentrations. The absence of a control group and the small number of dogs included in the study are potential limitations of this study.

In a second study, Panciera et al evaluated 8 adult dogs prior to and after experimentally inducing hypothyroidism with ^{131}I and subsequently during 5 weeks of levothyroxine supplementation.[73] Buccal mucosal bleeding time did not change throughout the study. No significant change in vWf:Ag concentration occurred in the dogs with the induction of hypothyroidism, but levothyroxine administration was associated with a decrease in vWf:Ag compared to healthy euthyroid control dogs.

Limitations of this study include the small number of study dogs, the relatively short period of time (23 weeks) that the dogs were hypothyroid prior to initiating supplementation, and the utilization of dogs with experimentally-induced, rather than naturally-occurring, hypothyroidism.

Based on the available information, the use of levothyroxine as a treatment for canine vWd remains controversial. While levothyroxine supplementation is advised for dogs confirmed to have hypothyroidism, supplementation is not expected to induce an increase in vWf:Ag concentrations in hypothyroid dogs.[72,73] However, whether levothyroxine will raise vWf:Ag levels in euthyroid dogs with low baseline values remains undetermined. Early studies suggested that BMBT may also improve with levothyroxine supplementation of hypothyroid dogs, but Panciera et al did not find any difference in the BMBT of dogs with induced hypothyroidism without vWd before or after supplementation.[67,73] However, the effect of levothyroxine on BMBTs in dogs with vWd remains undetermined.

If levothyroxine effectively increases plasma vWf:Ag concentrations and improves bleeding times in dogs with vWd, it would be an attractive therapeutic option. Levothyroxine is inexpensive and dogs are relatively resistant to side effects.[74,75] Potential side effects of thyrotoxicosis include tachycardia, tachypnea, hyperthermia, hyperactivity or lethargy, vomiting and diarrhea, weight loss despite polyphagia, polydipsia, polyuria or ECG changes.[76-80] Rapid metabolism and excretion of thyroid hormone by the liver and kidney may account for the low incidence of canine thyrotoxicosis.[74,81] As well, in plasma most T3 and T4 is protein-bound and only free hormone interacts with tissue receptors. Because only 1-30% of serum protein-binding sites are normally occupied, excess T4 is readily bound and cannot exert a physiologic effect.[81]

D. Hemostatic disorders and thyroid disease in humans

1. Hypothyroidism and hemostatic parameters

Hemostatic parameters, other than vWf, may also be altered in humans with hypothyroidism. Humans with moderate hypothyroidism have decreased fibrinolytic

activity, as reflected by low concentrations of D-dimers, increased α-2-antiplasmin activity, and increased t-PA and plasminogen activator inhibitor type 1 (PAI-1).[82] In contrast, humans with severe hypothyroidism have higher D-dimer concentrations, lower α-2-antiplasmin activity, t-PA and PAI-1 levels.[82] These findings suggest an increased capacity for fibrin degradation and may explain the increased tendency for bleeding in hypothyroid patients.[82] The increased fibrinolytic activity in hypothyroid patients is reversed by thyroid hormone supplementation.[83] Decreased hepatic synthesis has been proposed as a possible mechanism for low levels of coagulation factors VII, VIII, IX, XI, and XII and vWf in hypothyroidism.[83]

2. Hyperthyroidism and hemostatic parameters

Hyperthyroidism may be associated with a hypercoagulable state which results in serious arterial thromboembolic complications in some humans.[83,84] The mechanism of hyperthyroid-associated hypercoagulability remains incompletely understood, but several possibilities have been proposed. Endothelial cells may play a central role, possibly being activated and thus synthesizing proteins, including vWf.[85] The stimulus for endothelial activation has been investigated and possibilities include direct activation due to a hyperdynamic cardiovascular state, activation by the adrenergic nervous system, or activation mediated by cytokines. An alternate theory is that hyperthyroidism results in a state of reduced fibrinolysis which predisposes to hypercoagulability.[86,87]

Endothelial cells are metabolically active tissues that synthesize and secrete a variety of proteins.[85] Several endothelial derived proteins are increased in human hyperthyroidism including vWf, fibronectin, endothelin-1, PAI-1, thrombomodulin, and tissue factor pathway inhibitor.[85,86,88-92] Some of the serum protein elevations, including vWf, fibronectin, and PAI-1, have also been shown to return to normal following treatment of the hyperthyroidism.[85] In addition, thyroid hormone has been shown *in vitro* to upregulate messenger ribonucleic acid (mRNA) of vWf, fibronectin and endothelin-1.[88] Elevations of endothelial derived proteins in hyperthyroid humans suggest endothelial cell dysfunction, which may relate to the development of thromboembolic complications.[87]

It has been suggested that in hyperthyroidism, endothelial function and gene expression may be directly stimulated by increased shear forces on the vessel wall.[84] Hyperthyroidism results in a hyperdynamic circulatory state that may augment the vascular shear forces.[84] Excess thyroid hormone results in decreased vascular resistance and tachycardia and thus increased cardiac output.[74,77,93] There is an overall increase in blood volume mediated by increased erythropoietin production in response to elevated oxygen requirements.[83] Increased blood volume increases right atrial pressure and therefore increases preload and cardiac output.[93] Elevations in cardiac output may produce increased shear force in the vessels, and based on *in vitro* studies, shear forces modulate the expression of endothelial genes and thus could affect the production of endothelial derived proteins.[94]

Another theory is that hyperthyroid-related hypercoagulability is mediated by the adrenergic nervous system. The autonomic nervous system is activated in hyperthyroidism and adrenergic stimuli have been shown to produce increases in serum concentrations of vWf, t-PA, and FVIII:C in humans.[3,84,95] Epinephrine infusions result in increased vWf concentrations in normal dogs, but not in vWd-affected dogs.[96] In some human studies β-blockers have prevented the increase of vWf and FVIII:C in response to epinephrine, suggesting the release of vWf and FVIII:C may be mediated by catecholamines acting via β2-adrenergic receptors.[84,95,97] Catecholamine levels in hyperthyroid humans have been measured and found to be normal or low and it has been suggested that thyroid hormone may increase the sensitivity of adrenergic receptors to catecholamines, possibly by increasing the number of β-adrenergic receptors.[84,93,97] Enhanced β-adrenergic stimulation may act as a mediator for increases in endothelial associated proteins in hyperthyroidism.[85] However, in humans with hyperthyroidism an enhanced sympathetic response has been difficult to document.[93] Furthermore cultured endothelial cells do not secrete vWf in response to exposure to epinephrine, and in some studies, the administration of β-blocking drugs has not prevented the increase in endothelial associated proteins.[84,85] The dose of propanolol used in these studies may

have been inadequate or the mechanism underlying endothelial activation may be non-adrenergic.[97]

Cytokines are proposed as possible mediators of endothelial cell activation. Elevations in vWf concentration are reported in response to interleukin (IL)-6 in humans.[98] Interleukin-11 increases vWf concentrations in mice, but not in cultures of human umbilical vein endothelial cells.[99] When human recombinant IL-11 was administered to dogs, increases in plasma vWf and FVIII:C concentrations and vWf mRNA were documented.[100] Long-term stimulation with IL-1 has been shown to result in increased release of vWf from endothelial cells in response to a second agonist.[101] Tumor necrosis factor-α (TNF- α) alone or in combination with interferon-γ (IFN- γ) upregulates endothelial cell GP Ibα mRNA and protein synthesis.[102] Glycoprotein Ibα can then support the attachment of vWf to endothelial cells.[102] Humans with hyperthyroidism have increased concentrations of TNF-α and its receptor, as well as increased concentrations of IL-5, IL-6, IL-8, and IL-12.[103-106] Furthermore, following treatment for hyperthyroidism, normalization of TNF-α, IL-6, IL-8, and IL-12 concentrations has been documented.[103,105,106] IL-2 and IFN-γ concentrations are lower in humans with hyperthyroidism compared to normal humans.[107] Hyperthyroidism may result in alterations in serum cytokine levels that stimulate endothelial activation and release of endothelial derived proteins. This may be the underlying cause of hyperthyroidism-associated hypercoagulability. However, other studies found no affect of hyperthyroidism on serum concentrations of TNF-α, IL-6 and IL-11, and the role of cytokines in hyperthyroidism-associated hypercoagulability remains speculative.[84,105]

Arginine vasopressin (AVP) is a proposed mediator causing elevation of vWf in hyperthyroidism. Infusions of AVP or its analogue desmopressin cause increased plasma concentrations of vWf in healthy humans and dogs.[19,49,85] Compared to euthyroid humans, hyperthyroid humans have higher levels of AVP, and these AVP values return to normal when the hyperthyroidism is treated.[85] Therefore, it is proposed that elevations in thyroid hormones elevate endogenous AVP concentrations, and this may result in hypercoagulability.

Other researchers have proposed that hyperthyroid-associated hypercoagulability may result from a decreased state of fibrinolysis in hyperthyroid patients. This theory is based on observations that hyperthyroid patients have decreased t-PA antigen and increased PAI-1 concentrations.[86] Although other research has demonstrated increased t-PA activity in hyperthyroidism, t-PA antigen is a more reliable marker of the impaired release of t-PA by endothelial cells.[84,86,87] It is suggested that hyperthyroid patients have a shift in the balance between coagulation and fibrinolysis with an increase in PAI-1, which results in reduced fibrinolytic activity.[86,87] This may be the underlying mechanism of hypercoagulability in hyperthyroidism.

The mechanism underlying hyperthyroid-associated hypercoagulability remains incompletely understood, although several possibilities have been proposed and investigated. It is possible that the mechanisms proposed above may occur concurrently. It is possible that one or more of these mechanisms may explain the observation in vWd-affected dogs that administration of levothyroxine not only increases plasma vWf concentrations, but also improves BMBT which is a measure of primary hemostatic function.

CHAPTER II: EFFECT OF LEVOTHYROXINE ADMINISTRATION ON HEMOSTATIC ANALYTES IN DOBERMAN PINSCHERS WITH VON WILLEBRAND'S DISEASE

A. Abstract

This study tested the hypothesis that levothyroxine supplementation acts to increase plasma von Willebrand factor (vWf) concentration and enhance vWf function. The effects of levothyroxine administration were evaluated in 8 adult, euthyroid Doberman Pinschers with plasma vWf concentration <30%, characteristic of type 1 von Willebrand's disease (vWd). Levothyroxine (0.04 mg/kg PO q 12 hours) and placebo were administered for 30 days in a 2-period, 2-treatment, double-blinded, crossover design with a 30-day washout period between treatments. Buccal mucosal bleeding time (BMBT), vWf concentration (vWf:Ag), vWf collagen binding activity (vWf:CBA), Factor VIII coagulant activity (FVIII:C), serum total thyroxine (T4), free thyroxine (fT4), 3,5,3'-triiodothyronine (T3), and thyroid stimulating hormone were measured on days 0, 2, and 30 of each treatment period.

The 8 dogs (1 male, 7 females) had a mean age of 3 years, with markedly low plasma vWf:Ag concentrations (mean = 8.9%; reference range 70-180%) and a proportional lack of vWf:CBA (mean vWf:CBA = 11.1%; reference range >70%). In contrast, all dogs had FVIII:C within the reference range. The BMBTs varied among dogs, with 4 of 8 having values < 5 minutes. The response to placebo versus active levothyroxine treatment revealed no significant differences between groups at days 0, 2, or 30 for BMBT, vWf:Ag, vWf:CBA, and FVIII:C. Serum total thyroxine, fT4, and T3 were significantly higher in the levothyroxine-treated group compared to the placebo group at days 2 and 30. Thyroid stimulating hormone was significantly lower in the levothyroxine-treated group compared to the placebo group at days 2 and 30. No clinical signs of hyperthyroidism were observed. Levothyroxine at 0.04 mg/kg caused laboratory evidence of hyperthyroidism but did not affect plasma FVIII:C activity and vWf:Ag concentrations or the vWf-dependent functional parameters of collagen binding and BMBT. The results

of this study do not reveal a direct action of levothyroxine supplementation on plasma vWf concentration or activity in euthyroid Doberman Pinschers with type 1 vWd.

B. Introduction

Qualitative or quantitative deficiencies of vWf result in the bleeding disorder known as von Willebrand's disease (vWd).[18] Von Willebrand's disease is the most common congenital bleeding disorder of dogs and humans.[5,6] It is recognized in over 50 dog breeds, as well as in mixed breed dogs.[5] Type I vWd involves a quantitative decrease in vWf with a normal multimeric pattern, and affects many breeds including the Doberman Pinscher, in which the prevalence of vWd is as high as 70%.[5] Von Willebrand's disease can range from a subclinical disorder to a severe bleeding diathesis.[68]

Buccal mucosal bleeding time (BMBT) is commonly used as a screening test for vWd in dogs and is considered to reflect *in vivo* primary hemostatic capabilities.[7,30,108] However, prolonged BMBT is not specific for vWd, as it is affected by deficiencies or abnormalities of platelets or fibrinogen.[30] The functional capabilities of vWf may also be assessed by evaluating vWf collagen binding activity (vWf:CBA) that tests the ability of vWf to bind canine collagen.[37] Decreased vWf:CBA is present in dogs with reduced quantities of high molecular weight multimers, the most hemostatically active form of vWf.[37] Plasma concentrations of vWf, regardless of biologic activity or multimeric pattern, is quantitated as vWf antigen (vWf:Ag) concentration. Von Willebrand factor antigen concentration is expressed as a % compared to a standard control plasma pool that is designated 100%.[5] Definitive diagnosis of vWd is based on a plasma vWf:Ag concentration <49%.[6] Dogs with vWf:Ag levels <30% are considered to be at increased risk for bleeding diatheses.[29]

Treatment options for vWd are limited and those available increase plasma vWf concentrations only transiently. Desmopressin (1-desamino-8-D-arginine vasopressin or DDAVP) is believed to release intracellular stores of vWf, but there is marked variation in response to this drug.[8] Alternatively, vWf can be administered in plasma transfusions, although repeated use of blood products is associated with an increased risk of adverse immunological reactions or volume overload.[5] Cryoprecipitate contains vWf

concentrated in a smaller volume, but limited availability of this product restricts its routine use.[22] Administration of oral levothyroxine has been described as a treatment for canine vWd, with elevations in vWf:Ag concentration occurring as quickly as 48 hours after initiating therapy.[9,68] However, these results are not substantiated and the use of levothyroxine for the treatment of vWd remains controversial. The objective of this study was to test the hypothesis that levothyroxine supplementation sufficient to induce mild hyperthyroidism increases plasma vWf:Ag concentration and enhances vWf function when administered to euthyroid Doberman Pinschers with vWd.

C. Materials and Methods

Dogs: Eight adult Doberman Pinschers (3 intact females, 4 spayed females and 1 intact male) with vWf:Ag levels <30% were studied. Ages of the dogs ranged from 7 months to 8 years (mean 3.4 years) and body weights ranged from 28.1-34.5 kg (mean 31.9 kg). Dogs were determined to be healthy based on history, physical examination, complete blood count (CBC), serum biochemistry profile, one-stage prothrombin time (OSPT), and activated partial thromboplastin time (aPTT). Dogs were determined to be euthyroid prior to initiation of the study by measurement of serum thyroxine (T4) and endogenous thyrotropin (TSH) concentrations. The study was approved by the Virginia Tech Animal Care and Use Committee.

Experimental Protocol: A double blind 2-period, 2-treatment cross-over design with a 1 month interval between treatments was used. Dogs in group 1 received levothyroxine[b] at a dose of 0.04 mg/kg (range of 0.035-0.04 mg/kg) PO q12 hrs for 30 days initially and dogs in group 2 received a placebo (lactose in a gelatin capsule PO q12 hrs for 30 days) initially. Levothyroxine and placebo were encapsulated so they were indistinguishable by visual inspection, and dogs were randomly assigned to the treatment groups. Study parameters were evaluated at day 0, 2, and 30 of each treatment period and included physical examination, CBC, serum concentrations of total thyroxine (T4), 3,5,3'-triiodothyronine (T3), free thyroxine by equilibrium dialysis (fT4), and TSH, plasma vWf:Ag concentration, vWf:CBA, and FVIII activity (FVIII:C), BMBT, and 6-lead electrocardiogram (ECG). At each evaluation, owners completed questionnaires

regarding development of signs of hyperthyroidism, specifically vomiting, diarrhea, polydipsia, polyuria, polyphagia, change in activity or behavior, or change in hair coat. Blood samples were collected approximately 4 hours post-treatment (levothyroxine or placebo). Plasma samples were collected by jugular venipuncture into tubes containing 3.8% trisodium citrate[c] and centrifuged within 5 minutes at 2000 x *g* for 20 minutes at 4°C. Plasma was harvested and stored in plastic vials at -70°C until assayed. Serum samples were similarly harvested after blood samples were allowed to clot at room temperature for 20 minutes. The BMBT was performed by a single investigator (JCH) by a previously described technique,[34,73] using a spring-loaded bleeding time device.[d] Electrocardiograms consisting of leads I, II, III, aVR, aVL, and aVF[e] were recorded with the dogs in right lateral recumbency. Owner compliance with treatment administration was evaluated by performing pill counts at the end of each treatment period.

Sample Analysis: Measurement of vWf:Ag concentration by ELISA, vWf:CBA, and FVIII:C activity were performed at the Cornell University Comparative Coagulation Laboratory by methods previously described.[37,109,110] Results are reported as percentage of normal concentration, with normal pooled canine plasma designated as containing 100% vWf:Ag and 100% vWf:CBA. Serum TSH was measured by an immunoradiometric assay,[f] and serum concentrations of T4 and T3 were measured by radioimmunoassays.[g] Serum fT4 was measured using a commercially available kit that utilized an equilibrium dialysis method.[h] All assays were previously validated for use in dogs.[37,109,110] The concentrations of T4, fT4, and T3 were determined in serum from 56 clinically normal dogs.[111] The normal ranges established by the laboratories were as follows: T4 15 to 41 nmol/L, T3 0.70 to 2.26 nmol/L, fT4 9 to 43 pmol/L, and TSH -0.01 to 0.43 ng/ml.[111,112]

Statistical Analysis: Results are expressed as means or geometric means with 95% confidence intervals. For each measured response repeated-measure ANOVA (RMANOVA) was used to test for the effects of treatment and time and treatment x time interaction. Analyses were performed using the SAS® System.[i] Log transformation was performed to stabilize the variance for T4, fT4, T3, and TSH; these are presented as

geometric means with 95% confidence intervals. Differences of $P < 0.05$ were considered significant.

D. Results

Two dogs had histories of abnormally heavy bleeding during estrus, one of which also had a history of mild hemorrhage during otoplasty. No other history of abnormal hemostasis was reported for any of the dogs. All dogs had otoplasty and tail docking procedures and four dogs had ovariohysterectomies. One dog had distraction and stabilization surgery for caudal cervical spondomyelopathy performed 2 months prior to enrollment. This dog prophylactically received DDAVP and cryoprecipitate at the time of surgery and had been treated with prednisone that was discontinued 1 month prior to enrollment in the study. This dog also had a grade I/VI systolic heart murmur. Echocardiogram performed prior to surgery, revealed the dog to have mildly elevated peak systolic left ventricular outflow tract velocity, consistent with mild aortic stenosis. Physical examinations were unremarkable in the other 7 dogs.

Results of CBC, including platelet estimates, for each dog at each time period were normal. Mild elevations of alanine transferase (ALT) activity were present in 4 dogs (90, 96, 147 and 209; reference range 13-88 U/L). Otherwise, results of biochemistries were within reference intervals. The dog with the highest ALT activity initially was re-evaluated after completion of the study, at which time ALT was within the reference range. No dog had a prolonged aPTT and only 1 dog had a prolonged OSPT (19.0; reference range 13-18 sec.). Seven dogs were determined to be euthyroid based on normal T4, fT4 and TSH measurements. One dog had a slightly decreased fT4. Thyroid function was determined to be normal in this dog based on a normal TSH response test. Serum T4 concentration 6 hours after administration of 0.1 IU/kg IV bovine TSH[j] was normal (>30 nmol/L). All dogs had normal cardiac rhythm and rate on ECG recordings.

Side effects attributable to hyperthyroidism were not noted by owners. There were no significant differences for mean serum concentrations of T4, fT4 or TSH between the levothyroxine-treated group and the placebo group at day 0 (Figures 1, 2, and 3, respectively). On days 2 and 30, the serum T4 concentration for each dog receiving

levothyroxine was above the reference range (Figure 1). On day 2 mean concentrations of serum T4 ($P<0.001$) and fT4 ($P<0.001$) were significantly greater and the mean serum TSH ($P<0.001$) concentration was significantly lower in the levothyroxine group (Figures 1, 2, and 3). Similarly, on day 30 the mean concentrations of serum T4 ($P<0.001$) and fT4 ($P=0.0051$) were significantly greater and the mean serum TSH ($P<0.001$) concentration was significantly lower in the levothyroxine group (Figures 1, 2 and 3). There was no significant difference between the levothyroxine group and the control group for the mean T3 concentration at days 0 and 30, but there was a significant difference at day 2 ($P=0.0436$) (Figure 4).

There was no significant difference between mean heart rate in the placebo group (112, 111.3, 107.8 beats per minute, respectively) and the levothyroxine group (109.8, 110.5, 110.5 beats per minute, respectively) at days 0, 2 or 30. There was no difference between mean body weight for the placebo group (32.3, 32.2 kg., respectively) and the levothyroxine group (32.1, 32.0 kg., respectively) at days 0 and 2, but there was a significant difference (32.4 kg. for the placebo group, 31.9 kg for the levothyroxine group) at day 30 ($P=0.0068$). Seven of 8 dogs lost weight during the levothyroxine treatment and 4/8 dogs lost weight during the placebo period. There was no significant difference in body temperature between the 2 groups (100.9^{o}F for the placebo group, 100.8^{o}F for the levothyroxine group) at day 30, but the difference was significant (101.5^{o}F for the placebo group, 100.5^{o}F for the levothyroxine group) at day 2 ($P=0.0149$) and approached significance (101.5^{o}F for the placebo group, 101^{o}F for the levothyroxine group) at day 0 ($P=0.0508$).

There was no significant difference between mean values for vWf:Ag, vWf:CBA, BMBT or FVIII:C at day 0, day 2 or day 30 (Table 1, Figures 5, 6, 7 and 8). At the start of the study, one dog had a normal BMBT (< 4 minutes) and the other 7 dogs had prolonged BMBTs.

Owner compliance was evaluated by pill counts at the end of each 30 day treatment period. Two owners failed to return containers and therefore compliance could not be evaluated. For the remaining 6 dogs, the mean number of levothyroxine capsules that

were not administered was 3 (range 0 to 8) and the mean number of placebo capsules not administered was 4 (range of 0 to 10).

E. Discussion

No evidence of quantitative (based on vWf:Ag concentration) or qualitative (based on vWf:CBA and BMBT) change in vWf was observed in euthyroid Doberman Pinschers supplemented with a supraphysiologic dose of levothyroxine. The authors selected this dose of levothyroxine (0.04 mg/kg PO q12hrs) as they hypothesized that dogs treated with this dose would develop mild hyperthyroidism. Hyperthyroidism in humans is associated with a hypercoagulable state.[83] Factors that contribute to hypercoagulability in hyperthyroidism are not fully understood, but proposed mechanisms include an overall increase in blood volume, increased hepatic protein synthesis, and increased vWf:Ag concentrations mediated by cytokine release or endothelial activation.[83,84,98] Levothyroxine supplementation produced hyperthyroidism based on elevated serum T4 and fT4 concentrations and decreased serum TSH concentrations.

The hyperthyroidism induced in dogs of this study was considered mild and subclinical based on the lack of significant evidence of adverse effects of hyperthyroidism on clinical parameters. Mild weight loss noted on day 30 during levothyroxine treatment was the only clinical evidence of hyperthyroidism. However, 1/8 dogs gained weight during the levothyroxine treatment period and 4/8 dogs lost weight during the placebo period, so this may represent normal variation in the dogs' body weights. Food intake and exercise were not regulated and although no changes were reported by owners, alterations in caloric intake or energy expenditure, independent of the hormone supplementation, could potentially account for some or all of this change.

A standard therapeutic dose of levothyroxine has been reported to control vWd-related bleeding and to increase plasma vWf concentrations, an effect which is reported to occur within 24 hours and to persist for several days.[9] Correction of prolonged BMBTs to normal range within 48 hours of levothyroxine administration to dogs with vWd and hypothyroidism has also been described.[67] These reports have given rise to the recommendation to administer levothyroxine to lessen or control vWd-related bleeding,

as well as for prophylactic administration prior to elective surgeries.[67] However, data supporting these recommendations has never been published. Avgeris et al supplemented 4 dogs with levothyroxine for 1 month and found vWf:Ag concentrations increased in all 4 dogs.[68] Two of those dogs had small increases in vWf:Ag concentration which could have been the result of *in vivo* variability or due to assay factors. However, 2 of the dogs had marked increases in their vWf:Ag concentrations.

Results of the study presented here do not support the above findings. There was no significant effect of levothyroxine supplementation on vWf:Ag concentration at either day 2 or day 30. Furthermore, there was no effect on vWf:CBA or BMBT, indicating that a qualitative improvement in vWf function did not occur. Our results are consistent with other studies evaluating treatment of hypothyroid and euthyroid dogs with levothyroxine.[71-73] Hypothyroid dogs with normal vWf:Ag concentrations treated with replacement doses of levothyroxine had a small but significant decrease in vWf:Ag concentration.[72] In a preliminary study, euthyroid and hypothyroid Doberman Pinschers of unspecified vWd status were treated with levothyroxine for 12 weeks with no change in plasma vWf:Ag concentration.[71] Our results support the findings in dogs with experimentally-induced hypothyroidism where no significant change occurred in BMBT with the induction of hypothyroidism or with subsequent levothyroxine supplementation. In addition, no change in vWf:Ag concentration occurred with the induction of hypothyroidism, but levothyroxine administration was associated with a decrease in vWf:Ag compared to controls.[73]

Although a small number of dogs were studied, the population was homogeneous, consisting of a single breed with severe vWf deficiency (presumably type I vWd). The double blind, 2-treatment, 2-period design was chosen to ensure appropriate control of any natural variation occuring over time. Dogs with vWf:Ag concentrations <30% were used because this degree of deficiency is associated with an increased risk of hemorrhage,[29] and therefore we hypothesized they would be the most likely to benefit from a new treatment option.

In this study, levothyroxine was administered for 1 month. While it is possible that a longer course of therapy would have produced an effect, it seems unlikely since there was no trend for vWf:Ag concentrations or vWf:CBA to improve. As well, previous studies suggest a response to levothyroxine after 24-48 hours or by 1 month.[9,68]

In vitro assays, such as vWf:CBA, may not adequately represent *in vivo* hemostasis because they do not account for vascular shear forces. Evaluation of BMBT is considered by some to be the most reliable indicator of *in vivo* hemostatic capabilities, but the sensitivity of a comparable bleeding time test in humans has been shown to be as low as 50-65%.[7,30,39] Further studies utilizing an automated platelet function analyzer to mimic *in vivo* intravascular hemostatic function may be warranted. It has been proposed that the hemostatic improvement following levothyroxine supplementation may occur independently of alterations in vWf, possibly via alterations in platelet function, and platelet function analysis would permit evaluation of this hypothesis.[113]

Levothyroxine at 0.04 mg/kg q 12 hours caused laboratory evidence of hyperthyroidism but did not affect plasma vWf:Ag concentrations or FVIII:C activity and did not improve the vWf-dependent functional parameters of collagen binding and BMBT. The results of our study are clinically important, because they do not reveal a direct action of levothyroxine supplementation on vWf concentration or activity in euthyroid Doberman Pinschers with vWd. Based on this study, the administration of levothyroxine to Doberman Pinschers affected with vWd does not increase vWf plasma concentrations or improve vWf-related hemostatic function.

CHAPTER III: CONCLUSIONS

This study demonstrated that euthyroid Doberman Pinschers with severe deficiencies in plasma vWf concentration had no quantitative or qualitative change in vWf following supplementation for 2 or 30 days with a supraphysiologic dose of levothyroxine. Levothyroxine-treated dogs developed mild subclinical hyperthyroidism characterized by elevated serum concentrations of T4 and fT4 and decreased serum concentrations of TSH and no clinical evidence of hyperthyroidism. Levothyroxine administration has been recommended as a treatment for vWd, with the intent to increase plasma vWf concentrations and enhance vWf function as measured by BMBT. The results of our study contradict this recommendation and do not support administering levothyroxine to euthyroid Doberman Pinschers with vWd.

Our study evaluated vWf both quantitatively (as vWf:Ag concentration) and qualitatively (as vWf:CBA and BMBT). Automated platelet analyzers are a convenient point-of-care instrument, which are sensitive to deficiencies of primary hemostasis, including vWd. Automated platelet analyzers are designed to mimic flow conditions present in vasculature and thus may be more informative than the vWf:CBA regarding *in vivo* primary hemostatic function. The BMBT is an evaluation of *in vivo* primary hemostasis, but automated platelet analysis is more sensitive than BMBT and unlike the BMBT, the automated analyzers do not have a subjective end point. The addition of automated platelet analysis may have provided additional objective information and could have been an informative addition to our study. Other potential areas for improvement in our study include increasing the number of subjects, more frequent evaluations particularly early in the treatment period, increasing the duration of test period, and using different doses of levothyroxine.

Future studies are warranted to investigate whether a different dose of levothyroxine increases vWf concentrations or vWf activity. Furthermore, Doberman Pinschers with less severe deficiencies in vWf concentrations may have a response to levothyroxine that was not observed in these vWd-affected dogs, and investigation of this hypothesis is warranted.

FOOTNOTES

[a]PFA-100, Dade Behring Inc., Miami, FL

[b]Soloxine®, Daniels Pharmaceuticals, St. Petersburg, FL

[c]Vacutainer®, Becton Dickinson, Franklin Lakes, NJ

[d]Simplate®R, Organon Teknika, Durham, NC

[e]PageWriter XLi M1700A Cardiograph; Hewlett Packard

[f]Coat-A-Count TSH IRMA, Diagnostic Products Corporation, Los Angeles, CA

[g]Coat-A-Count Canine T4 and Canine T3 IRMA, Diagnostic Products Corporation, Los Angeles, CA

[h]Free T4 by Equilibrium Dialysis, Nichols Diagnostics, San Luis Obispo, CA

[i]SAS 8.02, SAS Institute Inc., Cary, NC 27513

[j]Sigma Chemical Co., St. Louis, MO

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APPENDIX I: FIGURES

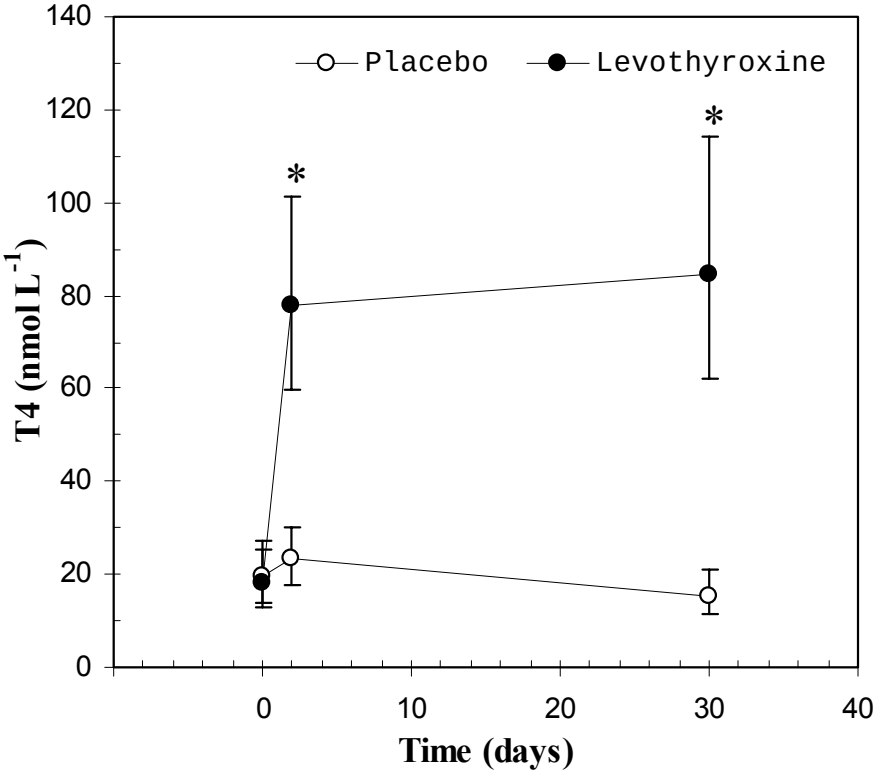

Figure 1. Mean serum total thyroxine (T4) concentration in Doberman Pinschers with von Willebrand's disease.
Mean serum total thyroxine (T4) concentration at day 0, 2 and 30 for 8 von Willebrand's disease-affected Doberman Pinchers treated with oral levothyroxine (0.04 mg kg^{-1}, q 12hr) and placebo (4 hr. post-treatment). Vertical bars represent the 95% confidence interval of the mean value. Significant differences are marked with an asterisk (*)($P<0.05$).

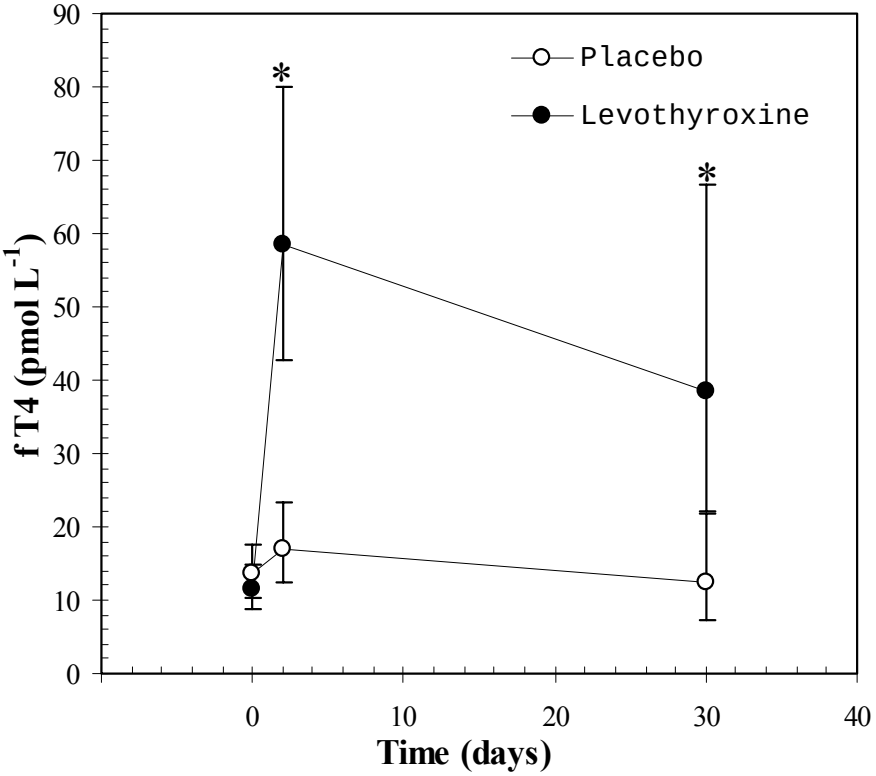

Figure 2. Mean serum free thyroxine (fT4) concentration in Doberman Pinschers with von Willebrand's disease.
Mean serum free thyroxine (fT4) concentration at day 0, 2 and 30 for 8 von Willebrand's disease-affected Doberman Pinchers treated with oral levothyroxine (0.04 mg kg^{-1}, q 12hr) and placebo (4 hr. post-treatment). Vertical bars represent the 95% confidence interval of the mean value. Significant differences are marked with an asterisk (*)($P<0.05$).

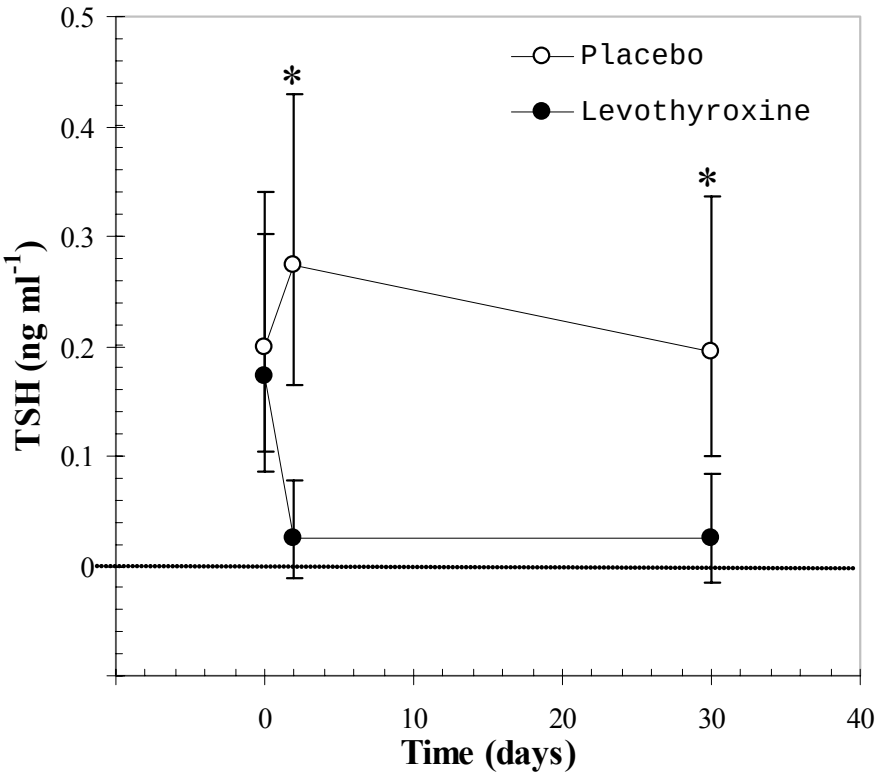

Figure 3. Mean serum thyroid stimulating hormone (TSH) concentration in Doberman Pinschers with von Willebrand's disease.
Mean serum thyroid stimulating hormone (TSH) concentration at day 0, 2 and 30 for 8 von Willebrand's disease-affected Doberman Pinchers treated with oral levothyroxine (0.04 mg kg^{-1}, q 12hr) and placebo (4 hr. post-treatment). Vertical bars represent the 95% confidence interval of the mean value. Significant differences are marked with an asterisk (*)($P<0.05$).

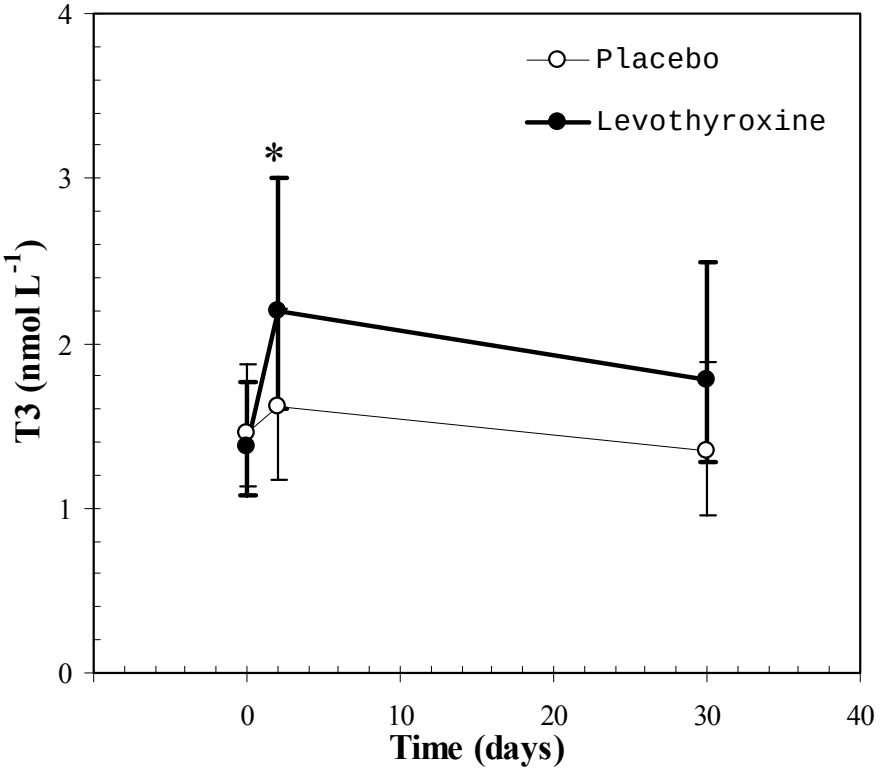

Figure 4. Mean serum 3,5,3'-triidothyronine (T3) concentration in Doberman Pinschers with von Willebrand's disease.
Mean serum 3,5,3'-triidothyronine (T3) concentration at day 0, 2 and 30 for 8 von Willebrand's disease-affected Doberman Pinchers treated with oral levothyroxine (0.04 mg kg^{-1}, q 12hr) and placebo (4 hr. post-treatment). Vertical bars represent the 95% confidence interval of the mean value. Significant differences are marked with an asterisk (*)($P<0.05$).

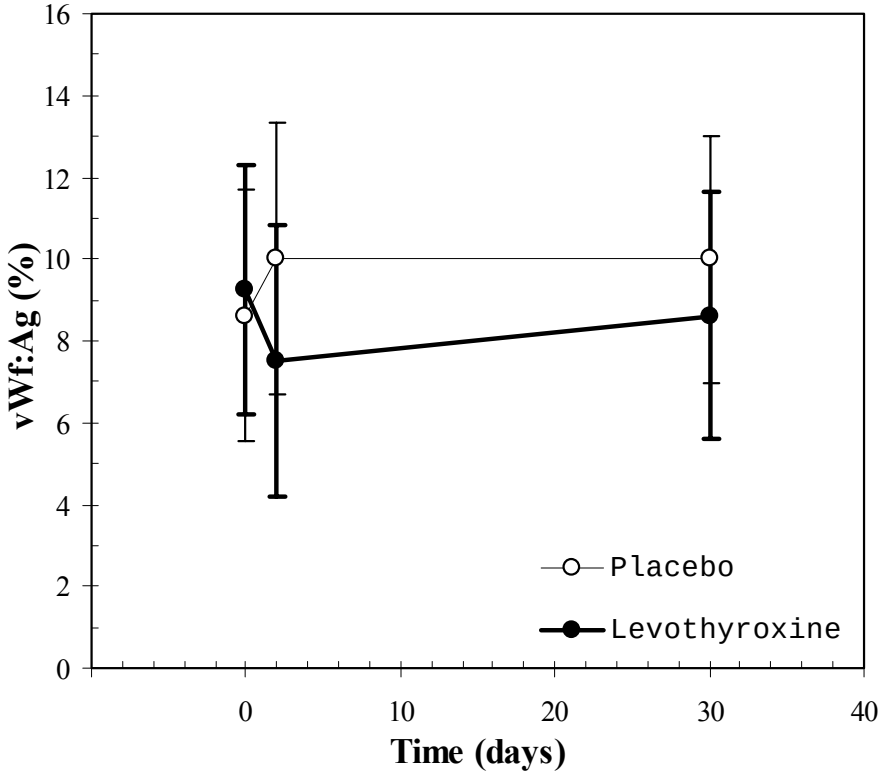

Figure 5. Mean plasma von Willebrand factor antigen (vWf:Ag) concentration in Doberman Pinschers with von Willebrand's disease.
Mean plasma von Willebrand factor antigen (vWf:Ag) concentration at day 0, 2 and 30 for 8 von Willebrand's disease-affected Doberman Pinchers treated with oral levothyroxine (0.04 mg kg^{-1}, q 12hr) and placebo (4 hr. post-treatment). Vertical bars represent the 95% confidence interval of the mean value. There were no significant differences ($P>0.05$). Reference range: 70% to 180% of a normal canine plasma pool.

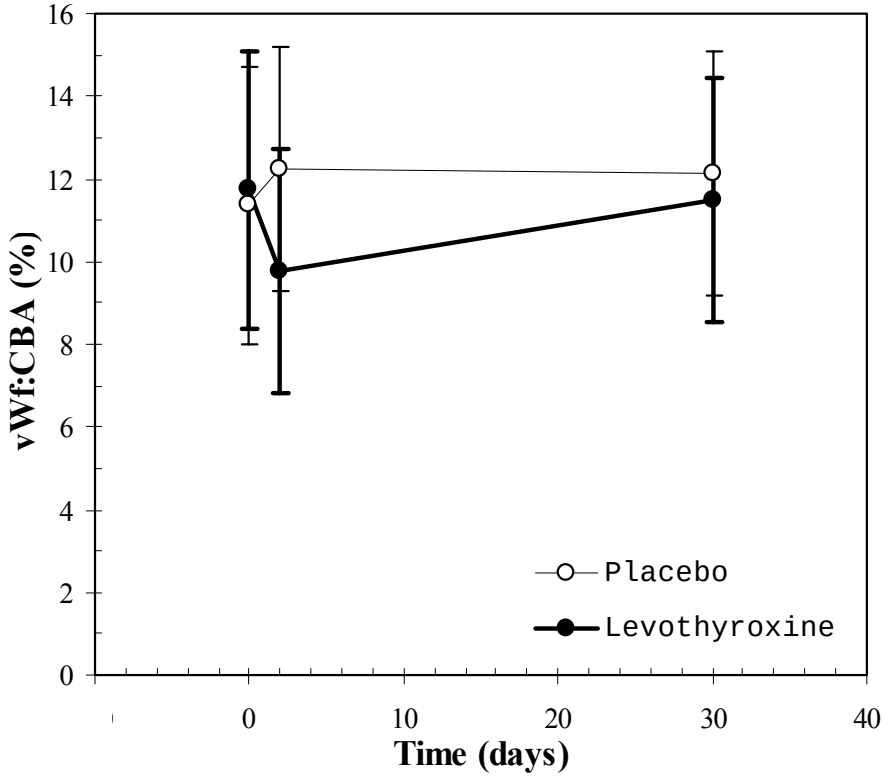

Figure 6. Mean plasma von Willebrand factor collagen binding activity (vWf:CBA) in Doberman Pinschers with von Willebrand's disease.
Mean plasma von Willebrand factor collagen binding activity (vWf:CBA) at day 0, 2 and 30 for 8 von Willebrand's disease-affected Doberman Pinchers treated with oral levothyroxine (0.04 mg kg^{-1}, q 12hr) and placebo (4 hr. post-treatment). Vertical bars represent the 95% confidence interval of the mean value.). There were no significant differences ($P>0.05$). Reference range: 68% to 143% of a normal canine plasma pool.

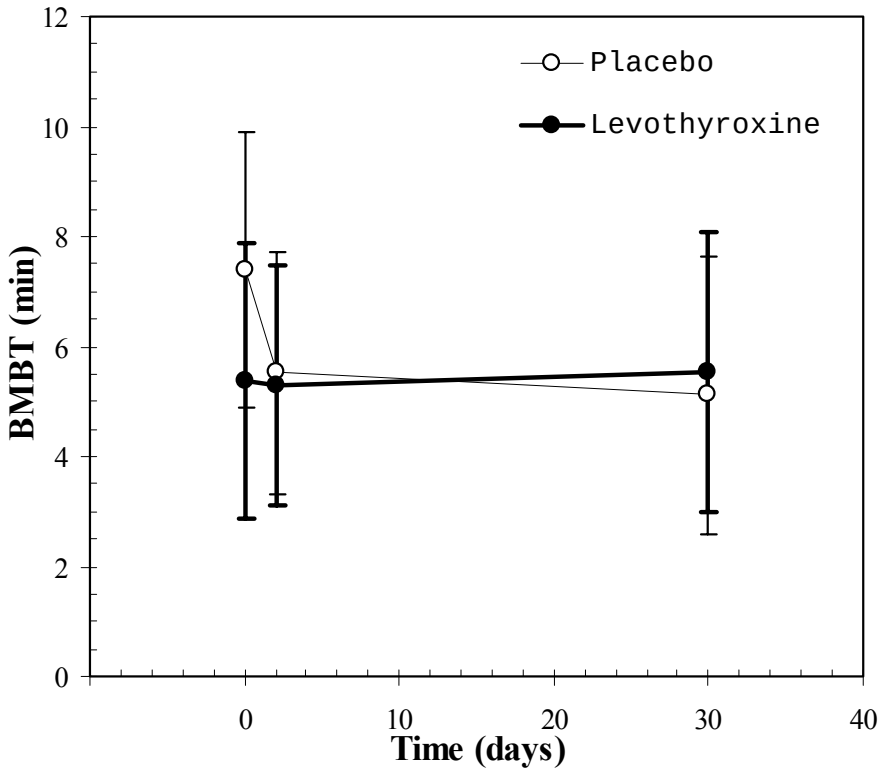

Figure 7. Mean buccal mucosal bleeding time (BMBT) in Doberman Pinschers with von Willebrand's disease.
Mean buccal mucosal bleeding time (BMBT) at day 0, 2 and 30 for 8 von Willebrand's disease-affected Doberman Pinchers treated with oral levothyroxine (0.04 mg kg^{-1}, q 12hr) and placebo (4 hr. post-treatment). Vertical bars represent the 95% confidence interval of the mean value. There were no significant differences ($P>0.05$). Reference range: < 4 min.

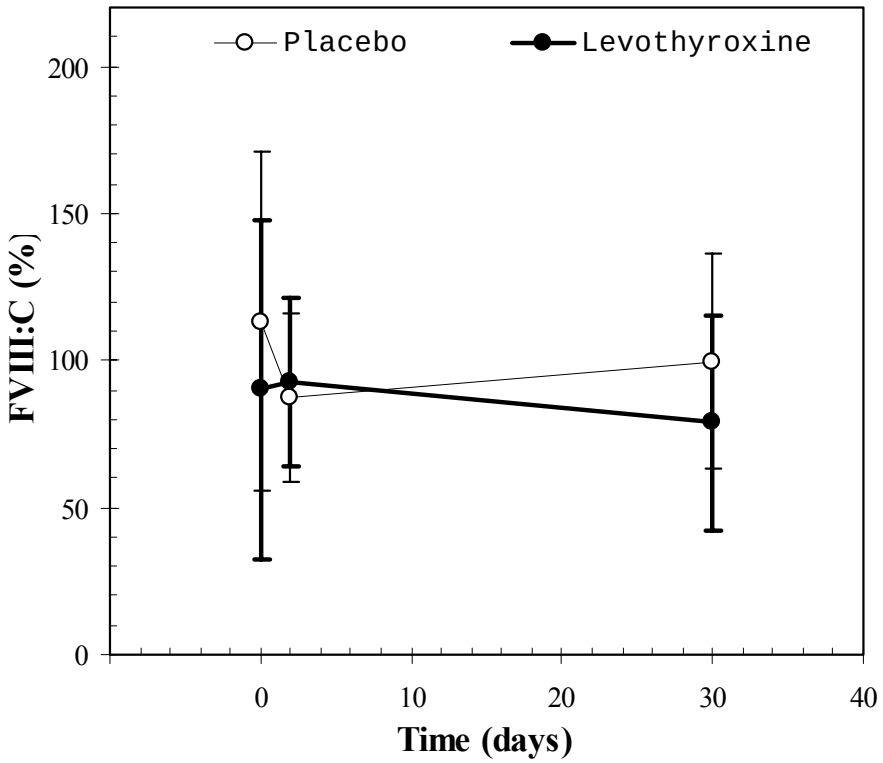

Figure 8. Mean factor VIII activity (FVIII:C) in Doberman Pinschers with von Willebrand's disease.
Mean factor VIII activity (FVIII:C) at day 0, 2 and 30 for 8 von Willebrand's disease-affected Doberman Pinchers treated with oral levothyroxine (0.04 mg kg^{-1}, q 12hr) and placebo (4 hr. post-treatment). Vertical bars represent the 95% confidence interval of the mean value.). There were no significant differences ($P>0.05$). Reference range: 50% to 200% of a normal canine plasma pool.

APPENDIX II: TABLES

Table 1. Mean plasma von Willebrand factor antigen (vWf:Ag) concentration, plasma von Willebrand factor collagen binding activity (vWf:CBA), mucosal bleeding time (BMBT), and factor VIII activity (FVIII:C) in Doberman Pinschers treated with levothyroxine and placebo.*

Variable (Normal Range)	Time (Day of Treatment)	Treatment	Mean	P-value	95% Confidence Interval	
					Lower limit	Upper limit
vWf: Ag (70% to 180%)	0	Levothyroxine	9.25	0.6478	6.18	12.32
		Placebo	8.63		5.56	11.69
	2	Levothyroxine	7.50	0.1307	4.19	10.81
		Placebo	10.00		6.69	13.31
	30	Levothyroxine	8.63	0.2944	5.62	11.63
		Placebo	10.00		6.99	13.01
vWf: CBA (68% to 143%)	0	Levothyroxine	11.75	0.8468	8.40	15.10
		Placebo	11.38		8.03	14.72
	2	Levothyroxine	9.75	0.1263	6.79	12.71
		Placebo	12.25		9.29	15.21
	30	Levothyroxine	11.50	0.6968	8.54	14.46
		Placebo	12.13		9.17	15.08
BMBT (< 4 min)	0	Levothyroxine	5.37	0.0754	2.87	7.88
		Placebo	7.40		4.90	9.91
	2	Levothyroxine	5.30	0.7412	3.13	7.47
		Placebo	5.53		3.33	7.73
	30	Levothyroxine	5.53	0.7189	3.00	8.06
		Placebo	5.12		2.59	7.65
FVIII: C (50% to 200%)	0	Levothyroxine	90.13	0.5432	32.57	147.68
		Placebo	113.12		55.57	170.68
	2	Levothyroxine	92.50	0.7374	63.88	121.12
		Placebo	87.63		59.01	116.24
	30	Levothyroxine	78.88	0.3410	42.33	115.42
		Placebo	99.50		62.96	136.04

* 8 von Willebrand's disease-affected Doberman Pinchers were treated with oral levothyroxine (0.04 mg kg^{-1}, q 12hr) and placebo. Samples were collected 4 hr. post-treatment at day 0, 2, and 30. There were no significant differences ($P>0.05$).

VITA

Johanna Heseltine was born in Saskatoon, Saskatchewan, Canada. She attended the University of Saskatchewan and graduated from the Western College of Veterinary Medicine (WCVM) in 1998.

Johanna completed a rotating small animal internship at the Atlantic Veterinary College in Charlottetown, Prince Edward Island. Upon completion of her internship, she returned to the WCVM as a clinical instructor in small animal medicine. Johanna is completing the requirements of a residency in small animal internal medicine and a Master of Science in Veterinary Science.