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Urinary 11-dehydrothromboxane B_2 concentrations in 20 dogs with primary immune-mediated hemolytic anemia

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Abstract

Background: Thromboembolic disease is a major cause of mortality in dogs with immune-mediated hemolytic anemia (IMHA). At present, no reliable biomarkers of individual patient thrombotic risk are available. In human medicine, increased urinary thromboxane concentrations have utility as markers of prothrombotic tendency in various situations.

Hypothesis/Objectives: First, to determine if urinary 11-dehydrothromboxane B_2 (u11-dTXB) concentrations are increased in dogs with primary IMHA compared to normal dogs; second, to assess whether u11-dTXB concentration is associated with survival, known prognostic indicators, or frequency of thrombosis in dogs with IMHA.

Animals: Twenty client-owned dogs diagnosed with primary IMHA and 17 healthy dogs volunteered by hospital staff.

Methods: Prospective case-control study. A previously validated ELISA was used to measure urine 11-dTXB concentrations, which were normalized to urine creatinine concentration (u11-dTXB:Cr). Samples were obtained at presentation from patients with primary IMHA. Standard clincopathological data at baseline and survival data were collected. Urinary 11-dTXB:Cr was compared between outcome subgroups, and correlated with known markers of disease severity.

Results: Baseline u11-dTXB:Cr was significantly higher in dogs with IMHA than in healthy dogs (median, 3.75; range, 0.83-25.36 vs 0.65; 0.24-2.57; $P = .003$) but did not differ between dogs with IMHA that survived and did not survive to 30 days after presentation, nor between dogs with and without clinical suspicion of thrombotic disease.

Conclusions and Clinical Importance: Urinary 11-dTXB:Cr is increased in dogs with IMHA compared to healthy controls, consistent with a prothrombotic state. However, in this IMHA population u11-dTXB:Cr was not associated with survival or suspected thrombosis.

KEYWORDS
canine, IMHA, survival, thromboembolism, thromboxane

Abbreviations: ACVIM, American College of Veterinary Internal Medicine; ALT, alanine aminotransferase; ASA score, American Society of Anesthesiologists score; CHAOS, canine hemolytic anemia objective score; CNS, central nervous system; CRP, C-reactive protein; CT, computed tomography; ECVIM-CA, European College of Veterinary Internal Medicine - Companion Animals; GDV, gastric dilatation volvulus; HCT, hematocrit; IMHA, immune-mediated hemolytic anemia; PLT, platelet count; PTE, pulmonary thromboembolism; T.bil, total bilirubin; TEG, thromboelastography; u11-dTXB, urinary 11-dehydrothromboxane B_2; u11-dTXB:Cr, urinary 11-dehydrothromboxane B_2-to-creatinine ratio.

1 | INTRODUCTION

Immune-mediated hemolytic anemia (IMHA) is a common autoimmune condition in dogs, and has a guarded prognosis with reported mortality rates of between 26% and 60%.[1-5] Thromboembolic disease is a major cause of morbidity and mortality in these patients, and dogs with IMHA are widely thought to be prothrombotic.[5-10] The pathophysiological mechanisms underlying the prothrombotic state in dogs with IMHA are still not fully understood, but studies have identified evidence of both hypercoagulability and platelet activation.[11-19]

There are 2 main approaches to assessing platelet activation: in vitro platelet function testing and measurement of markers of activation. These markers include changes in platelet surface molecule expression, platelet internal component density, and concentrations of substances released from platelets upon activation.[20-23] Thromboxane A_2 is released from activated platelets and plays a key role in amplifying platelet activation and initiating aggregation.[24-27] To date, studies assessing platelet activation in dogs with IMHA have focused largely on platelet function testing and laboratory measurement of platelet surface markers such as P-selectin.[11-13,15,28] The complexity of these techniques means they are largely restricted to use as research tools and are not available for routine clinical use.

Whereas thromboxane A_2 is highly unstable in serum, making measurement difficult, its urinary metabolites are more stable, making them potential biomarkers of platelet activation.[29-31] One such metabolite, urinary thromboxane 11-dehydrothromboxane B_2 (u11-dTXB), has been used in numerous pharmacological studies assessing the impact of antithrombotic and immunosuppressive drugs on platelet function in normal dogs.[32-37] In human medicine, urinary 11-dehydrothromboxane B_2-to-creatinine ratio (u11-dTXB:Cr) is a useful biomarker for the risk of thrombosis in conditions such as atherosclerosis, acute coronary syndrome, and stroke.[38-41] It also has been shown to be increased in patients with deep vein thrombosis and in conditions associated with an increased prevalence of thrombosis such as diabetes mellitus, chronic kidney disease, nephrotic syndrome, and inflammatory bowel disease.[38,42-47] In veterinary medicine, u11-dTXB:Cr has been shown to be increased in dogs with gastric dilatation volvulus (GDV), with higher postoperative ratios associated with increased postoperative complications.[48] It has not, however, been measured in patients with naturally occurring conditions associated with thrombosis, and in particular in dogs with IMHA. Our primary aim was to evaluate whether, consistent with increased platelet activation, u11-dTXB:Cr is increased at presentation in dogs with primary IMHA compared to healthy controls. A secondary aim was to assess whether in dogs with IMHA u11-dTXB:Cr at presentation is correlated with survival, known markers of disease severity, or occurrence of thromboembolism.

2 | MATERIALS AND METHODS

2.1 | Study design

u11-dTXB:Cr ratios were compared between dogs with primary IMHA and healthy controls by means of a prospective case-control study design. A power calculation was performed based on u11-dTXB:Cr ratios in a study of dogs with GDV compared to controls.[48] For an α of 0.05 and a β of 0.9, at least 6 dogs were required for each group. For the secondary hypothesis, that u11-dTXB:Cr would be higher in IMHA nonsurvivors than survivors, we aimed to recruit 20 dogs with IMHA, based on previously reported mortality rates of approximately 50%, to give 10 dogs in each group.

2.2 | Ethical considerations

The study was approved by the University of Glasgow School of Veterinary Medicine Research ethics committee (application 49a16).

2.3 | Criteria for IMHA case selection

Dogs presenting with suspected primary IMHA were prospectively recruited. Diagnostic criteria for IMHA were anemia (hematocrit [HCT] <37%), and the presence of 2 of 3 indicators of IMHA: spherocytosis, positive Coombs' test, and positive in-saline agglutination. A final classification of primary IMHA was reached on exclusion of possible triggers for secondary IMHA, with testing as deemed necessary by the primary case clinician. In all cases, diagnostic evaluation included abdominal ultrasonography and thoracic radiography, further biochemical analysis, urinalysis, and infectious disease testing based on travel history. This protocol is consistent with the recent American College of Veterinary Internal Medicine (ACVIM) guidelines for diagnosis of primary IMHA that were published after the inception of our study.[49] Hematology, serum biochemistry, C-reactive protein (CRP), and fibrinogen were measured at admission. Exclusion criteria included a final diagnosis other than primary IMHA, administration of antithrombotic medication before urine sample collection, or administration of immunosuppressive treatment for >48 hours at the time of presentation. Results of a CBC, serum biochemistry panel, CRP, and fibrinogen were recorded at presentation, along with spherocytosis as judged by a clinical pathologist, in-saline agglutination, and direct Coombs' test results. Urine samples collected for urinary thromboxane measurement also underwent biochemical analysis and sediment examination. The selected variables recorded for disease severity assessment included canine hemolytic anemia objective score (CHAOS), total bilirubin (T.bil), creatinine, urea, alanine aminotransferase (ALT), CRP, fibrinogen, HCT, platelet count (PLT), and American Society of Anesthesiologists (ASA) score at admission. Canine hemolytic anemia objective score is associated with survival at 30 days postpresentation and was calculated as described in previous studies[2,50] (Table 1). American Society of Anesthesiologists scores were determined after initial assessment either by the primary case clinician, or an emergency and critical care clinician assisting with case management. All therapeutic decisions were made independently by the primary case clinician; the study required no standardization of treatment. Treatment protocols and outcome measures were retrospectively extracted from the case records. Survival outcomes were categorized at hospital discharge, at 30 days after admission, and at

TABLE 1 Calculation of CHAOS illness severity score for dogs with IMHA

CHAOS criteria	
Age (y)	If ≥7 score 2, otherwise score 0
Temperature (°F)	If ≥102.0 score 1, otherwise score 0
Agglutination	If present score 1, otherwise score 0
Albumin (g/dL)	If <3.0 score 1, otherwise score 0
Bilirubin (mg/dL)	If ≥5.0 score 2, otherwise score 0
Total	Maximum score 7

Abbreviations: CHAOS, canine hemolytic anemia objective score; IMHA, immune-mediated hemolytic anemia.
Source: Adapted from Reference 2, based on Reference 50.

90 days after admission. When necessary, situational details regarding death or euthanasia were recorded for all cases. Records also were assessed retrospectively for any clinical suspicion of thromboembolic disease during the time between initial presentation and discharge from the hospital. This evaluation included macroscopic thrombi or evidence of organ infarction seen on imaging or at necropsy, unexplained tachypnea suspicious for pulmonary thromboembolism (PTE), and neurological signs suspicious for central nervous system (CNS) thrombosis.

2.4 | Control dogs

Control dogs were recruited via a request to the email list-serves of staff and students at the authors' institution. All dogs were deemed healthy based on verbal owner history, a health questionnaire, clinical examination, and routine biochemical testing and urinalysis, including sediment examination. Exclusion criteria included any abnormalities on history or clinical examination, a history of glucocorticoid, nonsteroidal anti-inflammatory drug or vaccine administration in the previous 2 weeks, and any history of long-term medications other than routine endo- and ectoparasiticides.

2.5 | Urine sample collection

Owners of control dogs collected voided urine samples within 24 hours of health screening. For IMHA patients, urine was collected within 24 hours of presentation by voiding unless cystocentesis was clinically required. Only patients with urine collected before antithrombotic medication administration were included. Urine samples were divided into 1 mL aliquots and frozen at −20°C within 24 hours of collection. Samples then were stored at −80°C for later batch analysis. Previous studies have shown no significant change in u11-dTXB concentrations with storage at room temperature for up to 6 days,[51] and long-term stability at −20 to −80°C.[30]

2.6 | ELISA protocol

Urinary 11-dTXB concentrations were quantified using a commercially available monoclonal ELISA validated for use with canine urine.[48] Urine samples were defrosted to room temperature and centrifuged at 2060*g* for 5 minutes to remove precipitated proteins before dilution. Initial samples were assayed, according to the manufacturer's instructions, at 3 dilutions. Once an approximate range for u11-dTXB in IMHA patients had been established, subsequent samples were assayed at 1:5 and 1:10 dilutions. All samples were assayed in duplicate. Results were determined from standard curves using commercially available software (Assayzap. Biosoft, Cambridge, UK). Results from replicates falling within the working range of the assay were averaged to give a final u11-dTXB concentration (ng/mL) for each urine sample. Any results falling outside of the linear region of the standard curve were discarded, and samples reassayed at 1:75 and 1:150 dilutions. The assayed concentrations were corrected for dilution and reported in pg/mL. The u11-dTXB concentrations then were normalized to urine creatinine concentration to give a u11-dTXB:Cr ratio. Urine creatinine concentration was measured at a reference laboratory by the Jaffe reaction method (Dimension Xpand. Siemens Medical Solutions) and reported in μmol/L, before conversion to mg/mL so as to report the final u11-dTXB:Cr as ng/mg creatinine as in previous studies.

2.7 | Statistical analysis

Data recording and statistical analysis were performed using commercially available spreadsheet and statistics software (Microsoft Excel, Windows 365, Microsoft, Redmond, Washington; IBM SPSS Statistics version 27, IBM Corp, Armonk, New York). Results were assessed for normality using Kolmogorov-Smirnov tests and visual inspection of normal Q-Q plots. Normally distributed data were compared using independent sample *t* tests, and non-normal data using Mann-Whitney tests. The u11-dTXB:Cr at admission was compared between IMHA and control groups, and between the IMHA subgroups for hospital discharge, 30-day survival, 90-day survival, and suspected thromboembolism. Variables previously associated with either disease severity or survival (CHAOS ≥3 and <3, T.bil, creatinine, urea, ALT, CRP, fibrinogen, HCT, PLT, ASA score at admission) also were compared between 30-day survival groups using Mann-Whitney tests for continuous variables and chi-squared tests for categorical variables. Where Mann-Whitney analysis identified a significant difference between groups, univariable binary logistic regression was used to further quantify the magnitude of effect. The u11-dTXB:Cr also was assessed for correlations with the same markers of disease severity by means of visual assessment of scatter plots, and linear regression analysis. Where the relationship between variables was nonlinear based on visual assessment of scatter plots, or residuals failed the assumptions of normal distribution and even variance based on histogram and pp-plot analysis, the variables were log-transformed to achieve a linear relationship.

3 | RESULTS

3.1 | IMHA dog population characteristics

Twenty-four dogs with suspected primary IMHA were recruited to the study. Two dogs were excluded because of alternative final

diagnoses: 1 with phosphofructokinase deficiency and 1 with IMHA secondary to neoplasia. Two additional dogs were excluded because urine was not collected within 24 hours of presentation. Twenty dogs with primary IMHA ultimately were included in the study. Seventeen of the 20 dogs fulfilled ACVIM consensus guideline diagnostic criteria for IMHA, whereas 3 fulfilled the supportive criteria[49] (Table S1). Affected breeds included 3 Cocker Spaniels, 2 Border Collies, 2 Springer Spaniels, 2 crossbreeds, and 1 each of Labrador retriever, Whippet, Bichon Frise, Cairn Terrier, Irish Setter, French Bulldog, Cockapoo, Miniature Schnauzer, Jack Russell Terrier, Labradoodle, and Pomeranian. The dogs ranged in age from 2 to 12 years (mean, 7.5 years). Selected clinicopathological data are shown in Table 2; additional data are shown in Table S2. All dogs were treated with glucocorticoids, 17 initially with IV dexamethasone, at a mean dosage of 0.32 mg/kg/d (range, 0.07-0.6 mg/kg/d) and 3 initially with PO prednisolone, at a mean dosage of 1.65 mg/kg/d (range, 1.01-2.0 mg/kg/d). Nineteen dogs received a second line immunosuppressive drug during initial hospitalization; 12 dogs received mycophenolate (mean dosage, 14 mg/kg/d; range, 8.1-24.3 mg/kg/d), 6 received cyclosporine (mean dosage, 8 mg/kg/d; range, 4.0-16.3 mg/kg/d), 2 received azathioprine (dosages, 2-2.1 mg/kg/d), and 1 dog received IV human immunoglobulin (0.5 g/kg). Overall, 1 dog was treated with a single immunosuppressive agent, 17 dogs were treated with 2 agents, 1 dog with 3 agents, and 1 dog with 4 agents. Clopidogrel was used for thromboprophylaxis in all dogs, at a mean dosage of 2.25 mg/kg/d (range, 0.97-4.7 mg/kg/d). Sixteen dogs received ≥1 blood transfusion; all were given packed red blood cells. Further information on exact drug combinations and dosages for individual patients is presented in Table S3.

3.2 | Healthy dog population characteristics

Seventeen healthy dogs were volunteered; 1 was excluded for otitis externa and osteoarthritic joint disease, leaving 16 control dogs eligible for the study. Several breeds were represented, of which 3 were Springer Spaniels. Ages ranged from 9 months to 11 years with a mean of 5.5 years. The mean age of the control dogs (mean, 5.5 ± 3.0 years) was not statistically significantly different from the IMHA dogs (mean, 7.5 ± 2.9 years; $P = .06$).

3.3 | Baseline urinary thromboxane results

Median baseline u11-dTXB:Cr of the IMHA group (3.75; range, 0.83-25.36) was significantly ($P = .003$) higher than that of the control dogs (0.649; range, 0.24-2.57) (Figure 1).

For the IMHA dogs, u11-dTXB:Cr was not correlated with any of the evaluated markers of inflammation and disease severity (CRP, HCT, PLT, CHAOS score, T.bil, creatinine, urea, ALT, total white blood cell count (WBC), fibrinogen, and ASA score) or age (Table 3).

3.4 | IMHA survival subgroup population characteristics

Of the 20 IMHA dogs, 5 died naturally whereas 4 were euthanized. Of the 4 that were euthanized, 2 were euthanized on day 1 or 2 postpresentation because of new neurological signs attributed to thromboembolic disease, whereas 2 were euthanized on days 5 or 10 postpresentation because of ongoing transfusion requirements. Eleven dogs (55%) survived to discharge, with a median hospitalization

TABLE 2 Selected biochemical variables for all 20 canine IMHA patients at baseline, IMHA patients surviving to 30 days postdiagnosis, and IMHA patients not surviving to 30 days postdiagnosis

	All IMHA dogs			IMHA 30-day survivors			IMHA 30-day nonsurvivors			
Parameter	n	Median	Range	n	Median	Range	n	Median	Range	*P* value
Age (y)	20	7.5	2-12	11	9	2-12	9	6	4-10	.34
CHAOS score points (maximum 7)	20	3.6	1-7	11	3	1-7	9	4	2-6	.24
T.bil (μmol/L)	20	137.4	0-667	11	27	4-143	9	294	0-667	.07
CRP (mg/L)	12	189	41-348	6	137	40-187	6	252	121-348	.03
HCT (%)	20	16	10-26	11	17	12-26	9	13	10-20	.03
PLT ($\times 10^{12}$/L)	19	324	101-846	11	333	101-846	8	314	125-499	.83
Fibrinogen (mg/dL)	15	592	368-777	8	625	368-718	7	572	388-777	1
Creatinine (μmol/L)	20	76	35-253	11	59	37-94	9	69	35-253	.18
Urea (mmol/L)	19	17	5-27	10	8.3	5-112	9	15.4	7-27	.02
ALT (U/L)	16	325	25-2704	9	36	25-237	7	171	32-2704	.04
ASA score	20	3.5	2-5	11	3	2-4	9	4	3-5	.002

Note: *P* values are for Mann-Whitney comparisons between 30-day survivors and nonsurvivors.
Abbreviations: ALT, alanine aminotransferase; ASA score, American Society of Anesthesiologists score; CHAOS, canine hemolytic anemia objective score; CRP, C-reactive protein; HCT, hematocrit; IMHA, immune-mediated hemolytic anemia; n, number; PLT, platelet count; T.bil, total bilirubin.

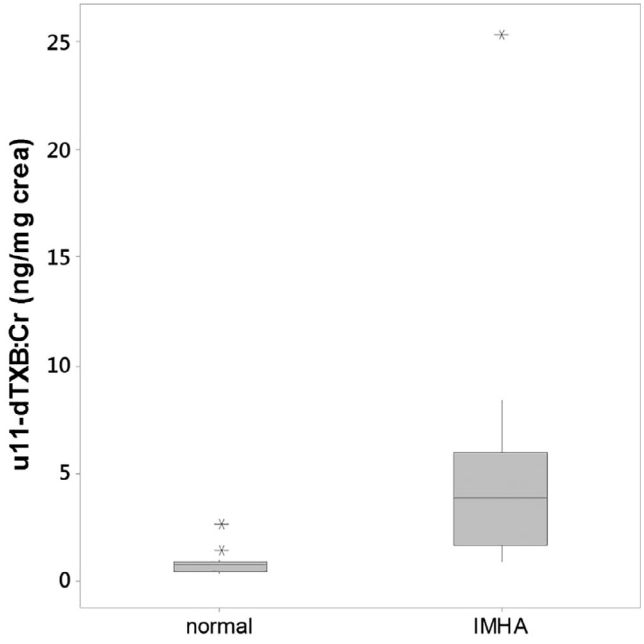

FIGURE 1 Urinary 11-dTXB:Cr ratios in 20 dogs with primary IMHA and 16 healthy control dogs. 11-dTXB:Cr, 11-dehydrothromboxane B_2-to-creatinine ratio; IMHA, immune-mediated hemolytic anemia

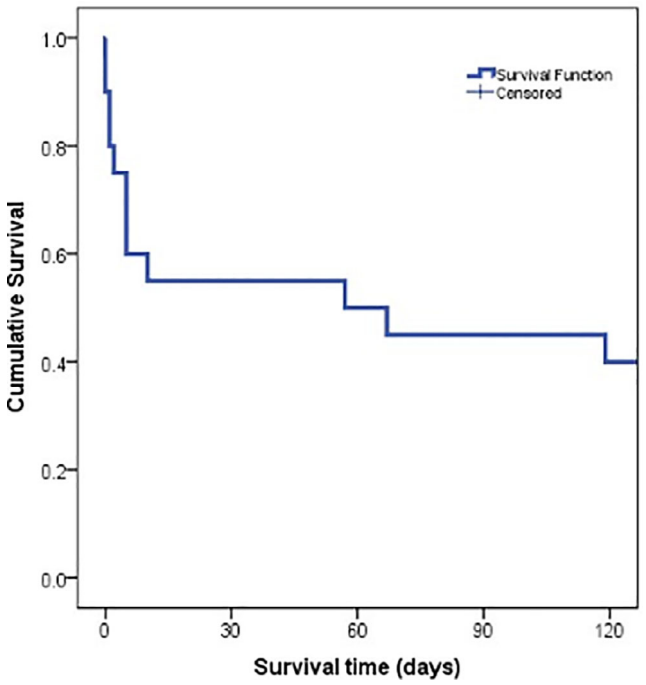

FIGURE 2 Kaplan-Meier survival curve up to 90 days postpresentation for 20 dogs with primary IMHA. IMHA, immune-mediated hemolytic anemia

TABLE 3 Results from linear regression analysis for selected markers of disease severity and urinary thromboxanes in 20 dogs with IMHA

			95% CI for β		
Variable	R^2	β	Lower	Upper	*P* value
Age	0.001	−.01	−0.16	0.14	.89
CRP	0.09	−.01	−0.009	0.007	.77
HCT	0.011	−.022	−0.122	0.079	.66
log(PLT)	0	.021	−0.71	0.752	.95
log(T.bil)	0.012	−.23	−0.3	0.3	.88
Creatinine	0.001	0	−0.007	0.008	.9
Urea	0.005	.11	−0.064	0.085	.08
log(ALT)	0.01	0	−0.001	0.001	.71
WBC	0.022	−.13	−0.55	0.3	.54
Fibrinogen	0.07	−.002	−0.006	0.002	.34

Note: All variables marked were assessed against log(u11-dTXB:Cr) to achieve a linear relationship for model validity, normal distribution of residuals and even variance of residuals.
Abbreviations: ALT, alanine aminotransferase; CI, confidence interval; CRP, C-reactive protein; HCT, hematocrit; IMHA, immune-mediated hemolytic anemia; PLT, platelet count; T.bil, total bilirubin; u11-dTXB:Cr, urinary 11-dehydrothromboxane B_2-to-creatinine ratio; WBC, total white blood cell count.

time of 8 days (range, 3-14 days). Two subsequently relapsed, 1 at day 57 and 1 at day 67 after presentation. The first was euthanized because of cost constraints and prognosis, whereas the second suffered cardiorespiratory arrest on presentation to the hospital after acute onset

TABLE 4 Binary logistic regression analysis for selected baseline variables with survival status at 30 days postdiagnosis for 20 dogs with primary IMHA

		95% CI for Exp(β)		
Variable	Exp(β)	Lower	Upper	*P* value
u11-dTXB:Cr	0.951	0.78	1.15	.61
CRP	0.067	0.998	1.068	.07
Urea	1.365	1.002	1.86	.05[a]
ALT	1.01	0.994	1.027	.23

Note: Exp(β) is the test statistic.
Abbreviations: ALT, alanine aminotransferase; CI, confidence interval; CRP, C-reactive protein; IMHA, immune-mediated hemolytic anemia; u11-dTXB:Cr, urinary 11-dehydrothromboxane B_2-to-creatinine ratio.
[a]Statistical significance.

of hemorrhagic diarrhea and neurological signs (necropsy was declined). Thus, the 30-day survival rate was 55%, equal to the discharge rate, and the 90-day survival rate was 45% (Figure 2). Individual patient survival data are presented in Table S4.

Of the disease severity markers, CRP, urea, ALT, and ASA score at presentation all were significantly higher in dogs that did not survive 30 days from presentation than in survivors. Additionally, HCT was significantly lower in 30-day nonsurvivors than in survivors (Table 2). The relationships were further investigated for CRP, urea, ALT, and HCT using binary logistic regression. Only urea retained statistical significance, with an increase of 1 mmol/L urea having an odds ratio of 1.36 for nonsurvival at 30 days (95% confidence interval [CI], 1.002-1.860; $P = .05$; Table 4). The proportion of dogs

with CHAOS score <3 and ≥3 also was compared between 30-day survivors and nonsurvivors, but the relationship was nonsignificant (chi-squared, $P = .19$).

3.5 | IMHA survival subgroup urinary thromboxane results

No significant difference was found in u11-dTXB:Cr at presentation between dogs with IMHA surviving to discharge (median, 2.20 ± 7.2; range, 0.83-25.36) and those not surviving to discharge (median, 4.29 ± 1.6; range, 1.71-6.28; $P = .45$). Identical results were found for dogs surviving and not surviving to 30 days after diagnosis, because all dogs that survived to discharge also survived to 30 days (Figure 3A). Also, no significant difference was found in u11-dTXB:Cr at presentation between IMHA dogs surviving to 90 days (median, 1.57; range, 0.83-8.37) and those not surviving to 90 days (median, 4.29; range, 1.71-25.36; $P = .22$; Figure 3B).

3.6 | IMHA thrombosis subgroup population characteristics

Five of the 20 IMHA dogs had a suspicion of thromboembolic disease during hospitalization. One dog suffered sudden respiratory arrest suspected to be a result of PTE, 2 developed neurological signs including nystagmus and seizures, and 2 dogs developed a combination of unexplained tachypnea and neurological signs. Of these dogs, 4 died or were euthanized at the time of the suspected thrombotic event, whereas 1 survived to discharge. Additional data on thrombus category for individual dogs are presented in Table S4.

3.7 | IMHA thrombosis subgroup urinary thromboxane results

No significant difference was found between the median u11-dTXB:Cr of the 5 dogs with suspected thromboembolism (4.29; range, 2.2-6.3) and the remainder of the IMHA group (3.48; range, 0.83-25.4; $P = .6$; Figure 4).

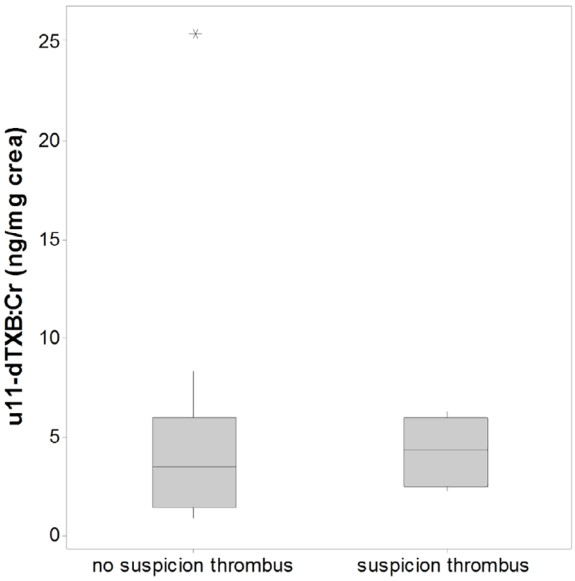

FIGURE 4 Urinary 11-dTXB:Cr for 5 dogs with IMHA and a clinical suspicion of thromboembolic disease during their hospitalization, and 15 dogs with IMHA without suspicion of thromboembolic disease. 11-dTXB:Cr, 11-dehydrothromboxane B_2-to-creatinine ratio; IMHA, immune-mediated hemolytic anemia

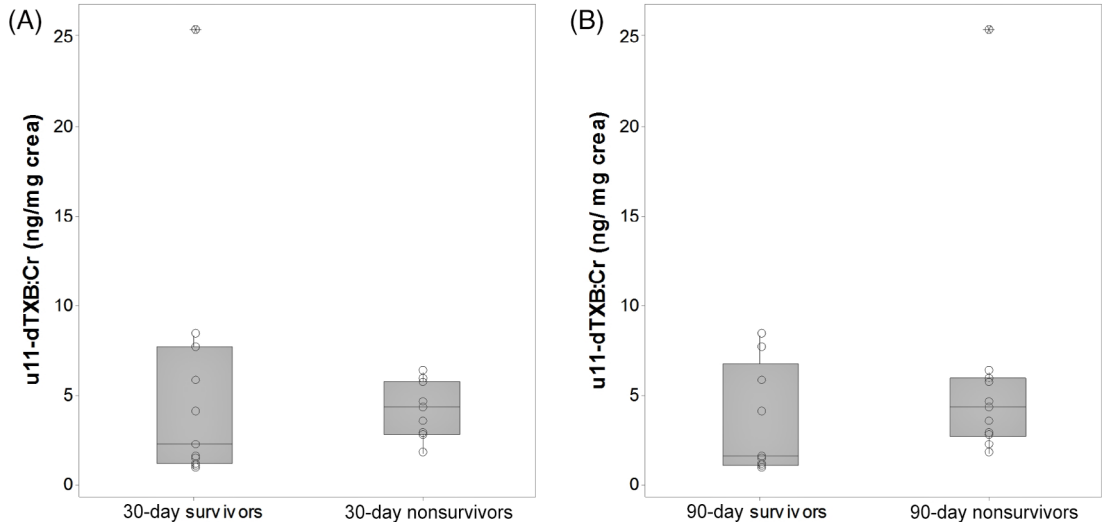

FIGURE 3 (A) Urinary 11-dTXB:Cr in 11 dogs with IMHA surviving to 30 days postdiagnosis, and 9 dogs with IMHA that died or were euthanized within 30 days of diagnosis. (B) Urinary 11-dTXB:Cr in 9 dogs with IMHA surviving to 90 days postdiagnosis, and 11 dogs with IMHA that died or were euthanized within 90 days of diagnosis. 11-dTXB:Cr, 11-dehydrothromboxane B_2-to-creatinine ratio; IMHA, immune-mediated hemolytic anemia

4 | DISCUSSION

Our main finding is that urinary 11-dTXB:Cr is significantly increased in dogs with primary IMHA at the time of initial presentation. However, no difference was found in the median u11-dTXB:Cr between IMHA patients surviving and not surviving to 30 days after diagnosis, or between patients with or without a subsequent suspicion of thromboembolic disease.

Thromboxane A2 is produced by activated platelets and by other cell types, including macrophages and endothelial cells during inflammation.[30,52-55] As well as being a product of platelet activation, it is itself a potent activator of platelets, acting via thromboxane receptors.[24-26] Although there is active debate over the relative importance of platelets in arterial vs venous thrombosis, both involve platelet activation to some extent. This involvement is believed to be either as the primary driver of thrombosis in the case of arterial thrombosis or secondary to thrombin generation from coagulation in the case of venous thrombosis.[7,15,56,57] As such, platelet activation is an important step in the general pathophysiology of thrombosis, and can be both the cause and consequence of increased serum thromboxane concentrations. Although we did not measure platelet activation directly, the high urinary thromboxane concentrations in the dogs with IMHA compared with the normal dogs is consistent with increased platelet activation in our study population, and thus potentially with a prothrombotic state. This finding is in agreement with previous studies showing evidence of increased platelet activation in dogs with IMHA including increased platelet P-selectin expression,[11] increased proportions of platelet-derived microparticles,[12] and decreased mean platelet component concentrations.[13,58] In human medicine, 11-dTXB is a well-established marker of in vivo platelet activation. It has been found to be increased in several prothrombotic conditions including stroke,[40,59] myocardial infarction,[39,41,60] and atherosclerosis,[38,52] and has been widely investigated as a surrogate marker for clinical response to aspirin.[51] As such, our findings provide additional evidence that IMHA is a prothrombotic condition and introduce u11-dTXB:Cr as a potentially useful biomarker for assessing thrombotic risk in dogs with IMHA.

Although we did not set out to investigate the source of increased u11-dTXB in our study, given that IMHA is an acute, highly inflammatory disease, it is likely that the u11-dTXB is not solely of platelet origin, but also from endothelial cells because of vasculitis,[52,53] and from inflammatory leukocytes such as macrophages.[54] In our study, no correlations between u11-dTXB:Cr and markers of inflammation such as WBC and CRP were found, nor with PLT or known markers of IMHA disease severity. As such, it could be argued that u11-dTXB:Cr is not simply reflective of the severity of inflammation or disease in individual dogs, and is not a reflection of platelet number. No other measures of platelet activation such as platelet surface markers were assessed in our study to evaluate for correlations with u11-dTXB:Cr, but regardless of source, increased u11-dTXB:Cr in this population of dogs likely reflects increased serum thromboxane concentrations and consequently increased platelet activation.

Interestingly, the range of u11-dTXB:Cr in the IMHA dogs overlapped substantially with that of the normal dogs. Because the range of u11-dTXB:Cr in the healthy dogs in our study is similar to that of the control group in previous studies, where the same units of measurement were used (range, 0.15-1.53 ng/mg creatinine),[32] this observation may suggest that not every dog with IMHA is prothrombotic at presentation. Alternatively, the prothrombotic tendency may not be primarily driven by platelet activation in all dogs with IMHA, and may be more a consequence of hypercoagulability in some individuals. This observation is consistent with dTXB:Cr recent thromboelastography (TEG) studies in dogs with IMHA in which hypo-, normo-, and hypercoagulable tracings have been reported.[18,19] Similarly, not all studies using flow cytometry analysis to evaluate platelet activation in dogs with IMHA found increased platelet activation in all cases.[12] Equally, it is likely the risk of thrombosis varies over the time course of disease. Serial TEG evaluations performed in dogs with IMHA showed that tracings became significantly more hypercoagulable over the first 5 days of treatment, but this observation may have been in part because of the impact of changing HCT because lower HCT has been shown to cause changes consistent with hypercoagulability on TEG.[18,61] Serial evaluation of u11-dTXB:Cr in future studies may provide additional useful insight into this variability. These observations raise the question of whether all dogs with IMHA are prothrombotic at presentation and require thromboprophylaxis as recommended in current treatment guidelines,[7,62] and emphasize the need for identification of reliable and accessible biomarkers of thrombotic risk to allow assessment of individual patients and individualized therapeutic intervention. As a biomarker, u11-dTXB:Cr has several advantages over other methodologies for assessing prothrombotic tendency, such as platelet function analysis, flow cytometry analysis of surface marker expression, or TEG, in that it requires no specialized equipment or storage requirements at the point-of-care, is noninvasive, and is stable in urine for up to 6 days at room temperature, making it feasible for samples to be sent to a central laboratory for analysis, and for owners to collect follow-up samples at home if required.

The secondary aims of our study were to evaluate if an association existed between urinary thromboxane and outcome in the IMHA patients, with the hypothesis that higher thromboxane concentrations would be associated with an increased risk of thrombosis. Because thrombosis is often difficult to definitively prove or exclude clinically and is a common cause of mortality and morbidity in IMHA patients,[5-10] we not only evaluated for an association with suspected thrombosis, but also for an association with survival and with known negative prognostic markers. We found no difference between baseline u11-dTXB:Cr in dogs with and without suspected thrombosis, and between survivors and nonsurvivors to hospital discharge as well as to 30 and 90 days after presentation. Also, no evidence was found for any association with any of the markers of disease severity. This observation may indicate that u11-dTXB:Cr is not a useful marker of thrombotic tendency in dogs with IMHA, and that thrombosis in these patients is driven more by activation of coagulation than by activated platelets. Previous studies have identified evidence of increased intravascular tissue factor expression, circulating procoagulant microparticles, decreases in plasma coagulation factor

concentrations, and the previously mentioned changes on TEG, all of which are consistent with hypercoagulability in dogs with IMHA.[13,16,18,19,28,63] Indeed, the recent ACVIM treatment consensus guidelines suggest that platelet activation in dogs with IMHA is probably secondary to thrombin generation, rather than a primary event, because venous thrombi, as usually seen in this disease, are typically fibrin-rich.[7] To date, however, none of these markers of hypercoagulability has been shown to be associated with either thrombosis or survival outcome. Our study was powered to detect a difference in u11-dTXB:Cr between healthy and IMHA dogs, rather than for the secondary measures of association with survival and thrombosis. As such, it is possible that the lack of association represents type 2 error because of small group sizes with our study being underpowered to detect smaller magnitude differences between subgroups within the IMHA population.

Other factors that may have impacted on our ability to detect an association between u11-dTXB:Cr and thrombosis include limitations in our ability to detect thrombi antemortem in our patients. Definitive diagnosis of thrombosis relies on a high index of clinical suspicion and confirmatory diagnostic imaging. In many cases, the advanced imaging required is both costly and requires general anesthesia, although with improvements in computed tomography (CT) technology, CT angiography to screen for PTE is becoming more feasible.[64] Existing ancillary tests such as D-dimers, fibrin degradation products, and TEG may in some cases increase suspicion that a thrombus is present, but have limited diagnostic utility because of poor specificity and only moderate sensitivity.[64-70] In our study, although all patients had abdominal ultrasound examination and thoracic radiography at presentation, no further imaging for suspected thromboembolic disease was performed in most cases. As such, subclinical thrombosis cannot be excluded in many of the surviving patients. Likewise, consent for necropsy was not given for any of the nonsurviving patients, and thus undetected subclinical thrombi cannot be excluded. Additionally, the 5 patients classified as having suspected thrombi were classified based on consistent clinical signs alone. None of the dogs suspected of having CNS thrombosis had confirmatory magnetic resonance imaging, nor did those with suspected PTE have CT angiography. Additional studies designed to detect thrombi at multiple time points, as well as additional measures of hypercoagulability and platelet activation, would be useful to investigate how u11-dTXB:Cr ratios correlate with individual patient thrombotic risk.

It is also likely that differences in treatment among individual dogs had a confounding effect on any relationship between u11-dTXB:Cr and outcome. All therapeutic decisions were made by case clinicians, as is normal hospital policy, with no attempt to standardize treatment as part of the study. Consequently, dogs received a range of immunosuppressive treatments, with variable drug combinations and dosages. Some medications used, such as glucocorticoids, aged packed red cells, human IV immunoglobulin, and cyclosporine may be prothrombotic, and administration of these after urine sample collection may have altered patients' thrombotic risk or survival, and thus confounded the relationship between u11-dTXB:Cr and these outcomes.[6,35,71-75] As such, it would be valuable to further evaluate u11-dTXB:Cr in future prospective studies assessing the impact of standardized treatments on outcome. Additionally, prior treatment (up to 48 hours) with immunosuppressive agents may have impacted u11-dTXB:Cr ratios. Cyclosporine reliably increases thromboxane synthesis and u11-dTXB:Cr in healthy dogs, whereas prednisolone had the same effect in some, but not all, dogs.[35,36,71] In our study, no dog at baseline had received cyclosporine, but glucocorticoids were administered in some of the dogs. Whether this had a confounding impact on the significance of baseline u11-dTXB:Cr for survival is unknown. Similarly, it is possible that differences in antithrombotic treatment started after sample collection masked any association between u11-dTXB:Cr at presentation and subsequent development of thrombosis. Although antithrombotic treatment was not standardized in our study, all dogs received clopidogrel alone as thromboprophylaxis. Our study was performed before publication of the ACVIM consensus statement, which suggests anticoagulant drugs as the initial choice for thromboprophylaxis in IMHA.[7] A range of clopidogrel dosages was used in our study, which may have variably impacted the risk of thrombosis in individual patients. The u11dTXB:Cr results were not available until several weeks after patient presentation and therefore had no impact on treatment decisions.

The inclusion of euthanized dogs in our study also may have affected the lack of association between u11-dTXB:Cr and survival. As in all veterinary studies assessing survival, euthanasia is an important confounder, and reasons for euthanasia are complex, involving owner ethical and financial considerations along with the clinical state and predicted prognosis of the individual patient. Of the 9 nonsurvivors, 4 were euthanized, of which 2 had developed severe neurological signs and death was considered inevitable. The other 2, however, were euthanized because of further transfusion requirements, and the possibility of survival with longer duration of treatment cannot be excluded. A further limitation of our study is that it was performed in a referral hospital. Although our findings of significantly different baseline CRP, HCT, urea, ALT, and ASA score between survivors and nonsurvivors at 30 days suggest our IMHA population is similar to those reported previously in the literature, it is also likely that our population does not reflect the full spectrum of disease severity, because more mildly affected dogs are less likely to be referred for tertiary care. As such, it is possible that future studies including a higher number of IMHA patients from both referral and primary care would better allow for evaluation of an association between u11-dTXB:Cr and outcome. Equally, although dogs in our study were diagnosed with primary IMHA based on clinical evaluation as deemed appropriate by diplomates of the European College of Veterinary Internal Medicine - Companion Animals (ECVIM-CA), variation existed in the level of screening performed, particularly for infectious diseases, and thus the possibility of undetected underlying diseases that could impact either u-11TXB:Cr or outcome cannot be completely excluded.

In conclusion, we identified increased u11-dTXB:Cr consistent with increased platelet activation in dogs with IMHA compared to healthy controls. Although u11-dTXB:Cr ratios at baseline were not significantly different between dogs surviving to 30 days and those not surviving, nor between dogs that went on to have suspected thrombi compared to those with no suspicion of thrombosis, small sample sizes and difficulties detecting thrombosis clinically limit the

reliability of these secondary findings. Additional studies focusing on the relationship between u11-dTXB:Cr and the clinical frequency of thrombosis in dogs with IMHA are warranted. Should u11-dTXB:Cr be a reliable biomarker of thrombotic risk, it may promote individualization of thromboprophylaxis in dogs with IMHA.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the University of Glasgow School of Veterinary Medicine Research Ethics Committee.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

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