Urinary Thromboxanes in dogs with conditions predisposing to thrombosis

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Abstract

A number of canine diseases are associated with an increased risk of thrombo-embolic disease at the population level. These include common conditions such as immune-mediated haemolytic anaemia (IMHA), protein-losing nephropathies (PLN), and protein-losing enteropathies (PLE). At present there is no reliable way of predicting which individual patients are at risk of thrombosis, nor do we know whether the risk to an individual remains constant over time or fluctuates throughout the course of their disease. One of the mechanisms implicated in prothrombotic states is increased platelet activation. There are several ways of measuring platelet activation, which can be grouped into two main categories: platelet function assays and markers of platelet activation. These platelet activation markers can either be molecules expressed on the platelet surface or substances secreted when platelets are activated. Thromboxane A_2 is one such substance. Serum levels of Thromboxane A_2 can be indirectly assessed via measurement of its metabolites in urine, including urinary 11-dehydrothromboxane B_2 (u-11dTXB). In humans, urine thromboxanes are used as biomarkers of thrombotic risk in several situations.

The aims of this study were threefold. Firstly, to assess whether urine thromboxanes are increased in dogs with IMHA, PLN and PLE. Secondly, to assess whether urine thromboxanes correlate with markers of disease severity, survival, or incidence of thrombosis in dogs with IMHA. And finally, to describe the change in urine thromboxane levels over the course of treatment of dogs with IMHA.

In this thesis I have shown that the u11-dTXB:Cr is increased in dogs with IMHA and PLE compared to healthy controls. I found no evidence of an association between u11-dTXB:Cr and markers of disease severity, survival or incidence of thrombosis in the subset of dogs with IMHA. The increase in u11-dTXB:Cr compared to healthy dogs was sustained over the first 6 weeks of treatment in the surviving dogs with IMHA. These findings have added to our knowledge of urinary thromboxanes as potential biomarkers of platelet activation in dogs with prothrombotic conditions.

Table of Contents

List of Tables

List of Figures

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Author's Declaration

The work presented in this thesis was performed solely by the author except where the assistance of others has been acknowledged

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List of Abbreviations

1.	11-dTXB	11-dehydrothromboxane B_2
2.	AchE	Acetylcholinesterase
3.	ACVIM	American College of Veterinary Internal Medicine
4.	ADP	adenosine diphosphate
5.	AIHA	autoimmune haemolytic anaemia
6.	ALT	Alanine aminotransferase
7.	aPTT	activated partial thromboplastin time
8.	ASA	American society of anaesthesiologists
9.	AT	antithrombin
10.	ATE	aortic thrombi
11.	Ca	Calcium
12.	CCECAI	Canine chronic enteropathy clinical activity index
13.	CHAOS	canine haemolytic anaemia objective score
14.	CI	coagulation index
15.	CIBDAI	Canine inflammatory bowel disease activity index
16.	CK	creatinine kinase
17.	CNS	central nervous system
18.	COX	cyclooxygenase
19.	CRP	C-reactive protein
20.	DIC	disseminated intravascular coagulation
21.	DVT	deep vein thrombosis
22.	FDPs	fibrinogen-degradation products
23.	HCT	Haematocrit
24.	IBD	Inflammatory bowel disease
25.	IMHA	immune-mediated haemolytic anaemia
26.	LTA	light transmission aggregometry
27.	MPC	mean platelet component concentration
28.	NETs	neutrophil extracellular traps
29.	PAR	protease-activated receptor
30.	PLE	protein losing enteropathies
31.	PLN	protein losing nephropathies
32.	PLT	Platelet count
33.	PS	phosphatidylserine

34. PSGL-1 P-selectin glycoprotein ligand-1
35. PT prothrombin time
36. PTE pulmonary thromboembolism
37. ROTEM rotational thromboelastometry
38. T.bil total bilirubin
39. TEG thromboelastography
40. TF tissue factor
41. TFPI tissue factor pathway inhibitor
42. TXA_2 thromboxane A2
43. TXR thromboxane receptor
44. u-11dTXB:Cr urinary 11-dehydrothromboxane B2-to-creatinine ratio
45. UPC urine protein-to-creatinine ratio
46. VTE Venous thromboembolism
47. vWF von Willebrand factor
48. WBA whole blood impedance aggregometry
49. WBC total white blood cell count

Publications and Presentations

Some of the work contained in this thesis has been the subject of the following publications or presentations:

Conference Proceedings

'Urinary 11-dehydrothromboxane B_2 levels in 20 dogs with primary immune mediated haemolytic anaemia.' Conway E A., Evans N., Ridyard A E. Oral Research Abstract Presentation. Proceedings of the British Small Animal Veterinary Association Congress, Birmingham, UK, 2019.

1 Introduction

Thrombotic disease, in which pathological blood clots form and obstruct blood flow to normal tissues, is a major cause of morbidity and mortality in human and veterinary patients. In human medicine, ischaemic cardiac disease due to atherosclerosis is the leading cause of death in the UK, and in 2010 cost the NHS an estimated £7,880 million[1]. Thrombosis is less common in dogs than in people, however there are number of disease states with known predispositions to thromboembolic complications. These well recognised prothrombotic diseases include immune-mediated haemolytic anaemia (IMHA), a condition involving destruction of red blood cells, protein losing enteropathies (PLE) such as inflammatory bowel disease, and conditions involving renal protein loss collectively known as protein losing nephropathies (PLN) (Goggs et al., 2019; Williams et al., 2017). It is likely that different pathophysiological mechanisms underly thrombosis in these different disease states, and also potentially between different individuals with the same disease. The three commonest types of thrombus in dogs are pulmonary thromboembolism (PTE), aortic thrombi (ATE), and thrombi of the brain causing neurological signs. Pulmonary thrombi cause obstruction of the pulmonary arteries, generalised hypoxia, respiratory distress, and can cause acute sudden death. Aortic thrombi tend to obstruct blood flow to the hindlegs and cause weakness or paralysis. While there are several veterinary studies identifying disease states that carry a risk of thrombosis, we do not currently have a means of establishing the level of risk in an individual patient. In a research setting, there are a number of ways of measuring platelet activation and hypercoagulability, but very few of these are easily available in the clinic. In an ideal world, we would be able to quantify the risk of thrombosis in an individual patient by means of a biomarker that is easily measured and minimally invasive to patients. In the human field, urinary thromboxanes have been widely investigated as markers of platelet activation and have shown promise as measures of thrombotic risk. To date, however, urinary thromboxanes have been only minimally investigated in veterinary patients.

Thus, the aims of this study were threefold. Firstly, to evaluate whether urinary thromboxanes are increased in dogs with three commonly seen prothrombotic conditions; IMHA, PLN and PLE. Secondly, since clinically we see the highest frequency of thrombosis in IMHA, to evaluate for any association between urinary thromboxane levels and disease severity, survival, or incidence of thrombosis. And finally, since the duration over which dogs with IMHA on treatment remain prothrombotic is unknown, to describe the pattern of urinary thromboxane levels in surviving dogs over the first 6 weeks of treatment.

[1] NICE Clinical guideline [CG181] Cardiovascular disease: risk assessment and reduction, including lipid modification Published date: July 2014 Last updated: September 2016

1.1 Normal Haemostasis

Haemostasis is the essential process by which haemorrhage from a vessel wall is stopped. The classical model of haemostasis separates the formation of a blood clot into three stages; Primary haemostasis in which an initial platelet plug is formed, secondary haemostasis in which a series of enzymatic reactions within the plasma leads to generation of fibrin to stabilise the platelet plug, and finally fibrinolysis in which the clot is dissolved once vessel healing is complete. While for ease these stages are classically thought of as occurring sequentially, in reality they occur simultaneously, with a fine balance between procoagulant, anticoagulant and fibrinolytic factors controlling normal clot formation. In recent years our advancing understanding of this process has led to increased appreciation of the complex interactions between the blood-borne factors and the surfaces upon which clotting occurs, including both blood vessels and a variety of blood-borne cells and particles. This has resulted in development of a new 'cell-based' model of haemostasis (Hoffman and Monroe, 2001; Smith, 2009). However the classical three part model of primary, secondary and tertiary haemostasis remains a useful starting point, as it underpins many of the clinically available tests of haemostasis (Weiss et al., 2010).

1.1.1 Primary Haemostasis

Primary haemostasis is defined as the formation of a loose platelet plug, which physiologically normally occurs at a site of endothelial injury. Certain pathological changes can also trigger primary haemostasis, leading to abnormal clot formation known as thrombosis. Primary haemostasis has three key stages; platelet adhesion, platelet activation and platelet aggregation. Although these are most easily thought of as occurring sequentially in an individual platelet, physiologically they will be occurring simultaneously, with the number of platelets involved governing whether a clot forms successfully. As well as the platelets themselves, von Willebrand factor released by endothelial cells, extracellular matrix proteins such as collagen, and plasma fibrinogen are all essential to primary haemostasis (McMichael, 2005; Weiss et al., 2010).

1.1.1.1 Platelet adhesion

Primary haemostasis is initiated when damage to the vascular endothelium results in two changes; exposure of subendothelial matrix proteins such as collagen to which platelets can adhere, and secretion of soluble mediators that contribute to platelet activation (McMichael, 2005; Smith, 2009; Weiss et al., 2010). In their circulating resting state, platelets constitutively express several surface receptors. These receptors include a family of integrins and glycoproteins that are key to platelet adhesion, and several families of receptors for soluble ligands such as prostacyclins and thromboxanes

which are key to platelet activation (Table 1). In their resting state, many of these receptors are only expressed at low levels, with greater numbers of receptors being trafficked to the cell surface after platelets have been activated. Some receptors also change to a higher affinity binding state once they have been activated, which helps to form more stable connections as clots mature (Bye et al., 2016; Goggs and Poole, 2012). Resting platelets are also continuously exposed to nitrous oxide and prostacyclins excreted by healthy vascular endothelium, which act on platelets to maintain them in their inactive state (Cheng et al., 2002). Once collagen and matrix proteins such as tissue factor (TF) have been exposed by endothelial damage, platelets can start to bind to the site of injury using these constitutively expressed surface receptors. The key receptors involved at this stage are glycoprotein VI and integrin GPIa-IIa (α2β1), both of which bind to collagen directly. Damaged endothelial cells also release von Willebrand factor (vWF) into the plasma and into the local subendothelial matrix. Platelets additionally bind to vWF via their surface receptors GPIIb-IIIa(αIIbβIII) and GPIb-IX-V so that vWF acts as an anchor between the platelets and the subendothelial matrix (Bye et al., 2016; Goggs and Poole, 2012). The importance of this second indirect binding via vWF is greater in arteries than in the small capillaries and veins, as the higher flow rate of arterial blood makes it harder for adequate direct binding to collagen to occur (Lenting et al., 2012). Binding to these receptors also activates intracellular signalling pathways that start to initiate platelet activation.

Table 1: Selected platelet surface receptors

Receptor	Other names	Main function	Main ligand
glycoprotein VI		direct adhesion	collagen
integrin GPIa-IIa	α2β1 integrin	direct adhesion	collagen
glycoprotein Ib-IX-V		indirect adhesion via vWF	vWF
integrin GPIIb-IIIa	αIIbβIII integrin	indirect adhesion via vWF platelet aggregation*	vWF fibrinogen*
thromboxane receptor family (TXR)		platelet activation	TXA_2
P2Y receptor family		platelet activation	ADP
thrombin receptor family	PAR	platelet activation	thrombin
P-selectin	CD62P	platelet aggregation platelet-leucocyte interactions	PSGL-1

Abbreviations: PAR, protease-activated receptor; vWF, von Willebrand factor; TXA2, thromboxane A2; ADP, adenosine diphosphate; PSGL-1, P-selectin glycoprotein ligand-1
Notes: *once receptor is activated

1.1.1.2 Platelet activation

Activation of platelets results in structural change and release of preformed surface receptors, coagulation factors and soluble molecules from cytosolic granules, all of which act together to recruit further platelets to the forming clot to cover the entire endothelial injury, and to stabilise the binding between the various clot components (Weiss et al., 2010). There are two groups of triggers for platelet activation; platelet adhesion and activation of surface integrin receptors as outlined above, and the action of soluble mediators. These mediators are excreted both by damaged endothelial cells and other nearby platelets that are already activated, and include ADP, thromboxane, adrenaline, prostaglandins, thromboglobulins, platelet factors, and thrombin. There are a number of different types of receptor for each of these mediators (Table 1), with each receptor linked to numerous interacting intracellular pathways. Two of the key ones are the phospholipase C pathway and the phospholipase A_2 pathway. The main results of the agonist pathways are an increase in intracellular calcium, or a decrease in cyclic AMP activity, which in turn lead to numerous end effects. Broadly speaking there are five key outcomes of platelet activation; structural change of the platelet; degranulation; alteration to exposed surface phospholipids; increased intracellular metabolic activity; and change of surface adhesion receptors to a higher affinity state (Bye et al., 2016; Goggs and Poole, 2012).

Firstly, the structural change to the platelets causes a change in platelet shape in such a way that increases their surface area, both improving stability of the clot, and providing a larger area of cell membrane for the procoagulant molecules involved in secondary haemostasis to work on. This shape change is mediated by the platelet cytoskeleton, which is made up of the contractile proteins actin and myosin (Bye et al., 2016; Goggs and Poole, 2012).

Degranulation is the second essential aspect of platelet activation. There are two types of granules contained within platelets; Alpha granules and Dense granules. The Alpha granules contain a mixture of adhesive proteins, soluble prothrombotic factors, and growth factors. The Dense granules contain further soluble prothrombotic factors and platelet agonists. Of the adhesive proteins contained within the Alpha granules, some such as fibrinogen are secreted into the plasma, others such as the GPIIb-IIIa receptor are trafficked to the platelet surface to be expressed, while some such as the glycoprotein P-selectin are both expressed and secreted (Rendu and Brohard-Bohn, 2001). P-selectin is a cell-adhesion molecule, with an important role in inter-platelet aggregation, helping to stabilise the initial binding between fibrinogen and platelet GPIIb-IIIa receptors, and so promoting formation of large platelet aggregates (Geng et al., 2012). P-selectin is also important in mediating interactions between platelets and white blood cells, so forming a key link between haemostasis and inflammation

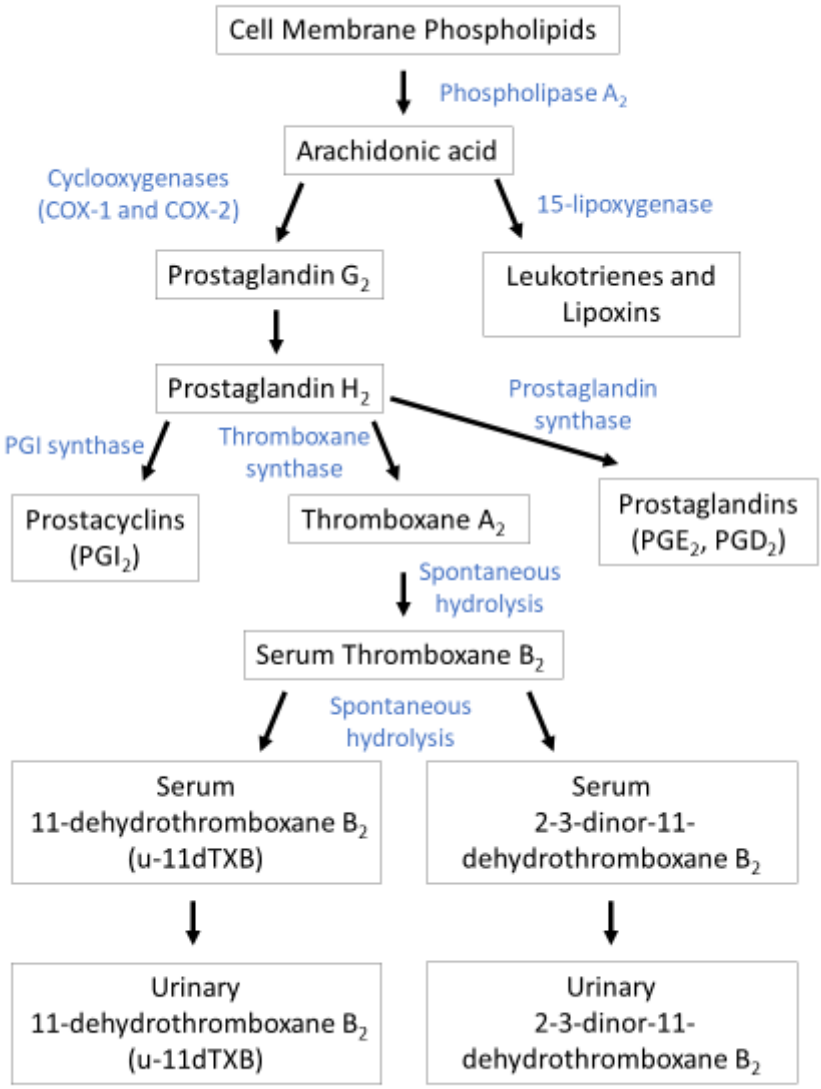
(Crockett-Torabi, 1998). The key soluble factors released pre-formed from the two types of granule include ADP, epinephrine, serotonin, histamine, calcium and certain clotting factors including V and XI. Of these, ADP is a particularly potent platelet agonist, and causes recruitment and activation of further circulating platelets. ADP acts through the P2Y family of receptors, which are G-protein coupled receptors constitutively expressed on the platelet surface. Of the family, the P2Y12 receptor plays a crucial role in amplifying platelet activation, as binding of ADP leads to further aggregation and granule release (Bye et al., 2016; Goggs and Poole, 2012; Murugappan et al., 2004; Rendu and Brohard-Bohn, 2001). Additional prothrombotic mediators are also created de-novo and released via increased activity of metabolic pathways within the platelets. The most important of these mediators is thromboxane, which is created from surface phospholipids via the phospholipase A_2 pathway (Figure 1). This pathway culminates with excretion of thromboxane A_2 (TXA_2) into plasma (Hamberg et al., 1975). There are a number of thromboxane receptors (TXR) making up the thromboxane receptor family, but broadly speaking activation of TXR leads to further platelet activation, structural change, and cytoplasmic granule release (Goggs and Poole, 2012; Murugappan et al., 2004).

Several key changes to the phospholipid platelet surface membrane also occur during platelet activation. One of these is membrane 'flipping', in which phosphatidylserine (PS), a negatively charged phospholipid usually expressed on the inner surface of the cell membrane, is flipped to the outer surface of the platelet (Goggs and Poole, 2012; Smith, 2009; Yamaji-Hasegawa and Tsujimoto, 2006). This has a number of actions, one of which is to make the overall platelet surface more negatively charged, which enhances binding of various coagulant molecules, including some of the clotting factor complexes involved in secondary haemostasis (Hoffman and Monroe, 2001; Smith, 2009). Some PS has also been shown to be shed from the cell membrane into the plasma in certain situations (Freikman et al., 2011). An additional membrane action that occurs following platelet activation is microvesiculation. This process causes blebbing of the platelet surface membrane, and release of small cell fragments known as microparticles. These microparticles express the same populations of receptors on their cell surface as the parent platelet and enhance haemostasis by acting as an additional surface on which the coagulation complexes can localise (Herring et al., 2013; Hoffman and Monroe, 2001; Melki et al., 2017; Smith, 2009). Both PS and microparticles are key to the cell-based model of haemostasis, as explored further below.

Finally, and most crucially for aggregation, platelet activation also causes a structural change in the surface integrin GPIIb-IIIa receptor. This conformational change can both be brought about by direct binding of vWF to the outer membrane surface of GPIIb-IIIa, or by increased intracellular calcium resulting from intracellular signalling downstream of other activated surface receptors acting on the intracellular domain of GPIIb-IIIa. This process of activation of GPIIb-IIIa by intracellular signalling

initiated by other receptors has been termed 'inside out' signalling and is key to understanding how triggers other than endothelial injury may lead to platelet aggregation and clot formation. Once activated, GPIIb-IIIa is able to bind to fibrinogen in addition to vWF. This step is crucial in enabling the platelets to bind not just to the damaged endothelium, but also to other activated platelets to form a stable clot (Goggs and Poole, 2012).

Figure 1: Phospholipase A2 Pathway



Abbreviations: COX-1, Cyclooxygenase 1, COX-2, Cyclooxygenase 2, PGI_2, prostaglandin I_2; PGE_2, prostaglandin E_2, PGD_2, Prostaglandin D_2, u-11dTXB, urinary 11-dehydrothromboxane B_2.

1.1.1.3 Platelet aggregation

Platelet aggregation occurs mainly through fibrinogen binding to the activated GPIIb-IIIa receptors on adjacent platelets, and acting as a bridge between activated platelets. As soluble fibrinogen circulates free in the plasma, platelet aggregation can occur readily once GPIIb-IIIa has been changed to its active form. Platelet aggregation results in a large loose clot of activated platelets held together by fibrinogen and to the damaged endothelial surface by vWF and direct integrin binding to collagen. These interactions are all relatively weak, however, and so while the primary platelet plug can rapidly limit haemorrhage, it is very easily disrupted until further stabilisation of the clot occurs via secondary haemostasis (Goggs and Poole, 2012; Weiss et al., 2010).

1.1.2 Secondary Haemostasis

Secondary haemostasis is the process by which the primary platelet plug is stabilised by fibrin, formed by the plasma-borne coagulation factors. These coagulation factors are a range of serine proteases and glycoproteins, the majority of which are synthesised in the liver. They are represented by Roman numerals in their inactive form, with an 'a' appended to indicate the active form. Classically, secondary haemostasis has been thought of as a cascade of enzymatic reactions, divided initially into two parts; the extrinsic and intrinsic cascades, which then both culminate in the common cascade. The extrinsic pathway is initiated by TF expressed by subendothelial cells, which binds to factor VII in the presence of calcium (Ca) and PS acting as cofactors. The activated factor VIIa-Ca-PS-TF complex is then able to activate factor X, starting the common pathway. The intrinsic pathway is initiated by surface contact of factor XII with exposed collagen. Factor XII then enzymatically activates factor XI, which in turn activates factor IX. The activated factor IXa, factor VIIIa, PS and Ca then form a complex which is able to activate factor X, again starting the common pathway. Once activated by either route, factor Xa then forms a complex known as the prothrombinase complex, which includes factor Xa, factor Va released from platelets, PS and Ca. The prothrombinase cleaves prothrombin to thrombin (also known as factor IIa), which in turn cleaves fibrinogen to give fibrin monomers. Thrombin is also responsible for activating factor XIII, which crosslinks the fibrin into polymers once it has been formed. Crosslinking of fibrin is essential to stabilise or mature blood clots (Weiss et al., 2010). Usually, in vivo, the extrinsic pathway is the predominant initiator of secondary haemostasis, with the intrinsic and common pathways amplifying the thrombin generation. It is important to note that thrombin can also activate factor XI of the intrinsic pathway directly, allowing it to amplify its own production significantly. The platelet surface, specifically the PS expressed on the surface of activated platelets, is also essential to this propagation of thrombin production. Binding to PS both enhances the formation and activity of the coagulation factor complexes, and helps to protect them from inhibition by natural anti-coagulant

molecules such as protein C and protein S (Hoffman and Monroe, 2001; Smith, 2009). These anticoagulant molecules are essential, however, in preventing inappropriate or excessive activation of coagulation under normal physiological conditions. Protein C and antithrombin (AT) are serine proteases that breakdown a number of the coagulation factors involved in the intrinsic and common pathways, while tissue factor pathway inhibitor (TFPI) performs a similar role in inhibiting the extrinsic pathway. Activation of these anticoagulant molecules is in turn linked to coagulation in a negative feedback loop; for example Protein C is activated by thrombin, using thrombomodulin as a cofactor (Kubier and O'Brien, 2012). Overall, the end result of secondary haemostasis is that the platelet plug formed in primary haemostasis is strengthened by deposition and crosslinking of fibrin amongst the platelets, to form a blood clot.

1.1.3 Tertiary Haemostasis

Tertiary haemostasis, also known as fibrinolysis, is the physiologic breakdown of the clot formed by the processes of primary and secondary haemostasis. Just as formation of the platelet plug and its stabilisation by fibrin are essential to stopping haemorrhage, fibrinolysis is equally essential to allow tissue healing and restoration of normal blood flow within the affected vessel. The key player in fibrinolysis is plasmin, another serine protease enzyme. Plasmin is produced by the liver as an inactive zymogen called plasminogen, which is released into the systemic circulation. Plasminogen then binds to fibrin at the site of clotting and is activated to plasmin by tissue plasminogen activator released locally by injured endothelial cells. Plasmin can then cleave both soluble fibrinogen, soluble fibrin, and crosslinked fibrin. Breakdown of the soluble fibrin and fibrinogen generates substances known as fibrin- or fibrinogen-degradation products (FDPs), while breakdown of cross-lined fibrin generates substances known as D-dimers. Detection of these various breakdown products has been investigated clinically for utility in diagnosing thrombotic disease (Birkbeck et al., 2019; Weiss et al., 2010).

1.1.4 The cell-based model of coagulation

While this three-stage classical model of haemostasis very effectively explains the basic processes of platelet aggregation, and stabilisation by fibrin, it is in a number of ways an oversimplification, particularly when it comes to the cascade model of secondary haemostasis. Clinically the cascade model is extremely useful, as the basic tests of secondary haemostasis available to practitioners can be divided into whether they reflect the intrinsic or extrinsic pathways. Physiologically, however, there are key interactions with other aspects of haemostasis that the cascade model misses out. The cell-based model of coagulation expands on this initial framework, incorporating not only more of the feedback loops between the three stages of haemostasis, but also interactions with the local microenvironment in which coagulation occurs, and so better reflecting true in vivo haemostasis. In

particular, recent research has further clarified the relationship between coagulation and the cellular surface on which it occurs, be that the endothelium or the platelet surface itself (Hoffman and Monroe, 2001; Smith, 2009).

These surface interactions can be both prothrombotic and antithrombotic. A key prothrombotic interaction is the need for cofactors for the majority of the clotting factors. These cofactors are commonly platelet surface molecules such as PS and TF, but also include calcium which is present in far higher concentrations in the local environment around activated platelets than it is in the general circulation. The majority of the clotting factors can perform their enzymatic reactions without their cofactors, but do so much more quickly when the cofactors are present. Some coagulation factor complexes, in particular the prothrombinase complex, can also only form effectively when bound to cell surface receptors. Being attached to a surface also offers the clotting factors a localised microenvironment that protects them from degradation by the blood-borne anticoagulants protein C, protein S, and AT (Hoffman and Monroe, 2001; Smith, 2009).

The cell-based model also helps to better incorporate the myriad of links between inflammation and haemostasis that have been discovered in recent years and are important when considering thrombosis in clinical disease states. Several studies have found evidence of increased TF expression on intravascular cells, including monocytes, which acts as a potent initiator of coagulation (Mackman, 2004; Piek et al., 2011). Particularly relevant are microparticles, small membrane derived vesicles free within the circulation. As outlined above, microparticles can be derived from activated platelets, but can also originate from activated endothelial cells, erythrocytes, and leucocytes in various inflammatory diseases. They can act as inappropriate initiators and accelerators of clotting by expressing certain surface markers such as PS and TF on their surface, even when vessel wall damage and associated haemorrhage are not present (Davizon and López, 2009; Herring et al., 2013).

As such it has become widely accepted that while the above 3 step division into primary, secondary and tertiary haemostasis is useful as an overview and for clinical diagnostics, it is very much a simplification. Physiologically these processes are occurring simultaneously and in a complex balance, with significant interactions with the vascular endothelium that are not reflected in the classical model. The novel concepts involved in the cell based model of coagulation have particularly influenced moves to develop new ‘global’ tests of haemostasis that encompass these cellular interactions, in contrast to the classical haemostatic tests which largely test the different blood-borne components in isolation.

1.2 Thromboembolic diseases

While clot formation can occur as a normal, essential physiological process at the site of vessel injury, it can also occur as a pathological process if it occurs in either an inappropriate place or in the absence of haemorrhage as an initiating factor. Pathological clot formation is termed thrombosis, with individual animals with an active tendency to form pathological clots described as being in a prothrombotic state. At the population level, certain diseases are recognised to carry an increased risk of thrombosis and may be referred to as 'prothrombotic diseases'. Pathophysiologically, in its most basic sense, thrombosis can be thought of as due to heightened primary or secondary haemostatic activity, or reduced fibrinolysis. Within these areas, however, there are multiple different abnormalities that may push the balance of prothrombotic and antithrombotic activity in the direction of thrombus formation.

1.2.1 Thromboembolic disease

Thrombosis is the pathological formation of a clot within a blood vessel, with thromboembolism referring to the process by which a clot dislodges from its initial site of formation, travels downstream in the circulation, before becoming lodged in a second location. This then causes a degree of obstruction to blood flow, and results in hypoxic injury to downstream tissues termed ischemia. It is important to note that thrombosis can occur in either the arterial or the venous sides of the circulation, with subtly different clot compositions, triggers for clot formation, and downstream sequelae (Weiss et al., 2010).

Thromboembolism is a major cause of morbidity and mortality in both humans and animals. In the human field, thrombosis of coronary arteries causing myocardial infarction is one of the leading causes of death in the developed world. Diseases such as deep vein thrombosis of the distal limbs, cerebral thrombosis causing strokes, and PTE causing respiratory distress are also significant causes of morbidity and mortality. In the US alone, 400,000 patients per year are affected by non-fatal PTE, while another 200,000 die from this disease (Long and Koyfman, 2017). In the veterinary field, distal aortic thromboembolism of the hindlimbs in cats is a common complication of cardiomyopathy, and one of the major causes of mortality in this condition. Although seen less commonly, aortic thrombosis in dogs can occur secondary to a number of different common conditions, including cardiac disease, neoplasia, hypothyroidism, hyperadrenocorticism, protein losing enteropathy, protein losing nephropathy, and diabetes mellitus (Williams et al., 2017). Thromboembolic disease including PTE is also a major cause of mortality in dogs with immune mediated haemolytic anaemia (IMHA) (deLaforcade et al., 2019; Kidd and Mackman, 2013; Swann et al., 2019). Clinically, some animals

present for veterinary care due to signs related to thromboembolic disease itself, rather than due to signs of the underlying disease process. This includes neurological signs due to cerebral or cerebellar emboli, tachypnoea or dyspnoea with PTE, and hindlimb dysfunction caused by aortic thrombi. Despite improvements in our understanding of the pathophysiology of thrombosis, however, in some studies up to half of dogs presenting with documentable thrombi have no detectable underlying predisposing condition (Winter et al., 2012). It is also important to note that not all thrombi are associated with clinical signs. In the human field, the prevalence of incidental emboli on thoracic and abdominal CT scans varies from 0.5-5%, while post mortem studies have identified incidental thrombi in between 9% and 63% of patients (Long and Koyfman, 2017). While no specific studies have been performed investigating the prevalence of incidental thrombi in veterinary patients, it is likely the prevalence is similar.

1.2.1.1 Pathophysiology of thrombosis

Broadly speaking, the pathophysiology of thrombosis is the same as physiological clot formation, but with an inappropriate trigger. In the 1800s the famous German physician Rudolf Virchow produced a body of work investigating the pathophysiology of thrombosis. He identified that predisposing factors could be grouped into three key areas, which have since become known as Virchow's triad (Bagot and Arya, 2008). These three areas are stasis of blood flow, endothelial injury, and hypercoagulability of the blood itself. While modern understanding of thrombosis has progressed, especially with regards to the underlying molecular mechanisms behind hypercoagulability, this key framework of thrombosis pathophysiology has endured.

In veterinary medicine, most research has focused on the abnormalities within the blood itself that may lead to hypercoagulability, and subsequently to thrombosis. These abnormalities encompass both increases in prothrombotic elements, and reduced antithrombotic elements. Some examples of the former include increased TF production, increased procoagulant microparticle levels, increased concentrations of soluble prothrombotic factors such as thromboxane and ADP, and increased activation of platelets (Jeffery et al., 2016; Kidd and Mackman, 2013; Piek et al., 2011; Shropshire et al., 2018). Reductions in anti-thrombotic mechanisms can include decreased levels of AT, thrombomodulin, protein C and TFPI.

The second corner of Virchow's triad, endothelial function, similarly normally exists in a balance of prothrombotic and antithrombotic activity. Increases in the prothrombotic activity, best exemplified by increased TF expression following injury, or decreased normal antithrombotic activity can promote thrombus formation. The normal antithrombotic activities of the endothelium include secretion of prostacyclin and nitric oxide (NO), both of which act directly on platelets to inhibit activation (Cheng

et al., 2002). Endothelial dysfunction alters this normal balance by reducing prostacyclin and NO excretion, and by increasing expression of prothrombotic surface molecules. Endothelial dysfunction can occur in various situations, most prominently in atherosclerotic plaque formation in people, but also in inflammatory diseases such as sepsis where vasculitis can occur (Poredoš, 2001).

The final aspect to Virchow's triad, stasis of blood flow, is harder to test for and quantify clinically, but equally important to thrombosis in certain situations. Specifically, blood stasis is extremely important in the pathogenesis of human deep vein thrombosis (DVT), which is commonly seen in patients immobilised in hospital for prolonged periods of time (Bates, 2012). In the veterinary field, blood stasis in the enlarged left atrium and auricle is thought to be a key mechanism behind thrombus formation in cats with cardiac dysfunction (deLaforcade et al., 2019).

Overall while abnormalities in all these three areas can inappropriately activate haemostasis and contribute to thrombus formation, differing mechanisms seem to predominately drive thrombosis in different underlying diseases. There are several diseases that we see a high incidence of thrombosis in in canine patients, the three of main relevance to this thesis being IMHA, PLN and PLE. Prior to looking at what is known of the specific pathophysiology of thrombosis in those three conditions, it is first relevant to outline how we detect thrombi in individual patients. Being able to reliably detect when thrombi are, and equally importantly are not, present in a patient is crucial to subsequently evaluating which predisposing abnormalities of Virchow's triad are present in patients with the same condition who do develop thrombi, compared to those who don't. Unfortunately, as outlined below, while the diagnosis of thrombosis and thromboembolism can be very straightforward, it can also be extremely difficult.

1.2.2 Detecting thrombosis

Clinically, thrombosis is often suspected in veterinary patients on the basis of clinical signs, which in turn vary depending on the site of thrombosis. The most commonly seen clinical signs of potential thrombi include sudden unexplained onset of respiratory distress, sudden onset neurological disease, or sudden onset of localised oedema. However these are all very non-specific. For example, in the case of PTE, the associated respiratory difficulty is often only attributed to PTE when other more easily detected cardiac and respiratory causes are excluded, and may only be suspected in dogs with conditions known or believed to predispose to thromboembolic complications. Equally, while most dogs with aortic thromboembolism (ATE) will present with hindlimb weakness, this can vary from being very mild exercise intolerance to hindlimb paresis or even complete paraplegia, with the severity of the clinical signs likely a reflection of the speed of onset of the clot. In studies of dogs diagnosed with ATEs, 15-47% present with acute signs, while 43-69% present with more chronic insidious onset

signs (Williams et al., 2017). Usually a combination of diagnostic imaging and laboratory tests are used to confirm a clinical suspicion of thrombosis, however the methods available have very variable sensitivities and specificities, as explored further below.

1.2.2.1 Imaging for detecting thrombosis

At present, diagnostic imaging is the only definitive way of confirming thrombosis in patients. The site of thrombosis does, however, have a significant impact on how effective imaging is in this role. The main groups of areas in which imaging may be used to detect thrombi are the large blood vessels in the abdomen and extremities, the pulmonary vasculature, the small vasculature within the central nervous system and finally the small capillaries in end-organs throughout the body.

In veterinary patients, when thrombi occur in the larger blood vessels such as the renal veins, splenic vein, femoral arteries or vena cava, they are often most easily visualised by means of ultrasonography. The use of Doppler flow can improve thrombus identification, since it allows evaluation of blood flow around the thrombus. In the human field, where deep vein thrombosis of the lower limbs are the commonest large-vessel thrombi, ultrasonography is also the first line test for detection and monitoring due to its easy accessibility (Karande et al., 2016). Contrast CT venography is, however, considered the gold standard with a sensitivity of between 71-100% and a specificity of 93-100% in meta-analyses. It is used particularly when the clinical index of suspicion is high, but ultrasound is negative. It is however much more expensive and invasive for the patient. Similarly, when assessing for portal vein thrombi in people, abdominal ultrasound is often used first, with a sensitivity of between 80-100%. CT and MRI can, however, provide additional information and help identify thrombi associated with malignancy (Keeling and Alikhan, 2013). Similarly, the use of MRI to detect aortic thrombi in dogs has also been reported however this is rarely used clinically due to the increased cost, time, and requirement for general anaesthesia compared to ultrasonography (Sharpley et al., 2009).

Pulmonary thrombi represent a particular diagnostic imaging challenge in both veterinary and human patients. In human medicine ventilation-perfusion scanning using nuclear scintigraphy was historically the gold standard for PTE detection. In recent years, however, the increasing availability and ease of use of CT scanners has made CT pulmonary angiography the current test of choice. Newer multidetector scanners have also enabled detection of thrombi in the subsegmental pulmonary vessels as small as 2 to 3mm diameter (Raja et al., 2015). CT pulmonary angiography has also recently been investigated for utility in identifying PTE in dogs. In a study of 12 dogs with IMHA suspected to have PTE based on unexplained respiratory distress, CTPA confirmed the diagnosis in four (33%) and was strongly suspicious for PTE in a further three (24%). The authors noted that while the sensitivity of CTPA in humans for PTE is high, between 83 and 100%, differences in CT slice thickness and patient

size mean that the sensitivity for dogs, particularly small dogs, is likely to be much lower. Given the lack of a gold standard for diagnosis of PTE, the actual diagnostic utility of CTPA could not be assessed, but was accurate in three dogs that underwent post-mortem examination. As such CTPA is a promising option for canine PTE detection in future, particularly as newer CT technology becomes available in the veterinary field.

By far the biggest challenge for detecting thrombi, however, is when they occur in the smallest venules, arterioles and capillaries in the end organs. In these locations the thrombi are not visible to ultrasonography, and so commonly only the end-organ effects of the thrombus are seen, such as focal splenic or renal infarctions. Similarly in the CNS, areas of cerebral or cerebellar ischaemia due to thrombi may be visualised on MRI, but the causative thrombi themselves are not usually visualised unless venous angiography is used (Weimar et al., 2012).

Overall while a positive finding of a thrombus on imaging is likely to be reliable, negative results are likely to be less so, due to low sensitivity for small clots. As such a number of laboratory tests have been developed to aid in the detection of thrombi. These tests are ideally used to increase or decrease the index of suspicion for a thrombus, so clinicians can decide whether further advanced imaging is likely to be useful.

1.2.2.2 The role of laboratory tests in detecting thrombosis

A number of the laboratory tests of clotting have been evaluated for their utility in helping to detect thrombosis. The classical tests of haemostasis; fibrinogen concentration, platelet count, Prothrombin time (PT) and activated partial thromboplastin tine (aPTT) have been found to have variable associations with the presence of thrombi and are all very non-specific. As such they are widely accepted to be unhelpful in screening for thrombosis, although they may have a role in detecting a prothrombotic or hypercoagulable state. As markers of clot breakdown, tests of FDPs and D-dimers were developed with the aim of being more sensitive for thrombosis, but clinically have been found to be poorly specific, and in many cases also poorly sensitive. Investigators have also started to explore whether the newer viscoelastic tests such as Thromboelastography (TEG) have any utility in thrombosis detection, with varying conclusions. Specific detail about TEG methodology and interpretation can be found in section 1.3.1.2 and in Figure 2.

D-dimers have been widely suggested to be a useful rule-out test for thrombi, on the basis that if a clot is present, some degree of fibrinolysis would be expected to be occurring. However clinically, the utility seems to vary depending on the location of the thrombosis. For screening for PTE in people, D-dimers have been found to have a negative predictive value of 99%, albeit with a poor specificity of around 41% (Perrier et al., 1997). As such, for PTE there are well recognised clinical algorithms in which

a positive D-dimer result in an at-risk patient acts as a trigger for further diagnostic imaging (Raja et al., 2015). However the same does not necessarily hold true for other clot types. A meta-analysis evaluating D-dimers for screening for human DVT found a similarly low specificity, but also found a lower than expected sensitivity, ranging from 51-100%, with a third of the studies reporting a sensitivity <90%. Overall the authors concluded that the use of negative D-dimers as a rule-out for DVT could not be recommended (Heim et al., 2004). In both situations, the low specificity means that positive D-dimer results may indicate the presence of a thrombus, but cannot be used as a diagnostic test alone. Fewer studies have been performed in the veterinary field, but on the whole they show similar results. Based on detection of clots at post-mortem, D-dimers have been shown to have a poor specificity of ~30%, but also a low sensitivity of 80-87% at clinically relevant cut-offs (Epstein et al., 2013). Authors of one study concluded that D-dimers could be used to exclude thrombi only at a level of 0 ng/ml (Nelson and Andreasen, 2003). Unlike in the human field, however, initial studies have failed to find an association between D-dimer levels and the presence or absence of PTE on CT angiograms (Goggs et al., 2014) Overall while D-dimers may have some utility in guiding the index of suspicion for thrombosis in people, the situation in dogs remains unclear.

The newer viscoelastic tests such as TEG have mainly been used to evaluate for hyper- and hypo-coagulability. A few studies have, however, looked for associations with the presence of thrombosis, but at present there appears to be no association between TEG variables and either thrombosis detected either post mortem, or PTE detected via CT angiography. In the first study looking for an association between TEG and subsequent post-mortem evidence of thrombosis, the authors found no statistically significant association between TEG variables and necropsy evidence of thrombosis. Of note however, at post mortem 75% of the thrombi found were deemed to be acute, 10% subacute, and 15% chronic, and the time between TEG and necropsy ranged from 1-7 days, so it is likely that there was significant variability between the time the TEG was performed and the time of clot formation in individual dogs, which may have impacted on the findings (Thawley et al., 2016). In another study comparing TEG findings in 6 dogs with PTE and 19 sick dogs without PTE, as determined by CT angiography, no consistent differences in TEG traces alone were found between the groups. Additionally, while some of the dogs with PTE had hypercoagulable TEG traces as was anticipated, some had normocoagulable and hypocoagulable traces (Marschner et al., 2017). It is important to note when evaluating TEG traces between studies, that there are a number of sources of variability in the methodology. Most notably, different activators, including collagen and kaolin can be used to start the in vitro clotting process, while some early TEG studies used no activators at all. While there are now consensus guidelines on how TEG should be run, there is still known to be significant inter-operator variability in results generated via the same machine, let alone between devices at different

centres (Goggs et al., 2014). As such at present, the utility of TEG for helping to guide the index of suspicion for the presence of thrombosis is unclear, but seems to be low.

1.2.2.3 Post mortem thrombosis detection

It is important to note when evaluating the utility of these different tests for detecting thrombosis, that there is currently no reliable gold standard against which to compare. Although many studies use post-mortem as the gold standard for evidence of thrombosis, or absence thereof, there are some noteworthy limitations to this approach. Even in dogs undergoing a full post-mortem examination thrombosis can be difficult to determine definitively, both due to dissolution of ante-mortem clots, and due to formation of post-mortem clots. Post-mortem fibrinolysis has been shown to start occurring as quickly as 3 hours after death in dogs. Additionally, smaller clots in minor vasculature may not always be found. For example, in one study of 29 dogs with PM confirmed pulmonary thrombi, 4/19 dogs had only very small thrombi present, which required extensive dissection to detect, and could have been easily missed (Moser et al., 1973).

In conclusion, a diagnosis of a thromboembolic event in veterinary patients is most commonly a suspected rather than confirmed diagnosis, based on a combination of clinical signs, presence of underlying risk factors, and imaging findings. In humans, unlike in dogs, there are some defined guidelines for when to screen patients for thrombosis, using combinations of laboratory testing and diagnostic imaging, however the starting point is always a set of suspicious clinical signs, as blanket screening is likely to produce large numbers of false positive results. As yet, while similar schemes have been suggested for PTE in dogs, they are not yet validated, and overall testing for thrombosis is very much down to individual clinician judgement.

1.2.3 Diseases with an increased prevalence of thrombosis

As mentioned previously, there is an increased prevalence of thrombosis in dogs with certain diseases. For the purposes of this thesis these diseases will be referred to as ‘prothrombotic diseases’, although it is important to note that an individual animal with a ‘prothrombotic disease’ may not have an actively increased risk of thrombosis at all timepoints of its disease course. Equally not all individuals with the same prothrombotic disease may be at equal risk of thrombosis, due to the numerous different pathophysiological mechanisms that influence haemostasis, which will each be abnormal to varying degrees in individual animals. Conditions considered to be prothrombotic in veterinary medicine include IMHA, PLN, acute necrotising pancreatitis, hyperadrenocorticism, certain neoplasias, some cases of sepsis, PLE, and cardiac disease in cats but not in dogs (deLaforcade et al., 2019). Of these conditions, three have been selected as the focus of this thesis, for various reasons. IMHA has by far the highest prevalence of thrombotic complications and is a very common cause of referral of

animals to referral hospitals for care. There is also an extensive pre-existing body of work suggesting that platelet activation is important in the pathogenesis of thrombosis in IMHA. Equally both PLN and PLE, while associated with a lower prevalence of thrombosis, are common diagnoses for patients seen through the internal medicine service. In contrast to IMHA, however, little work has been done investigating platelet activation in thrombosis in these patients, but newer studies from similar diseases in the human field raise the question of whether the role of platelets has historically been underappreciated.

1.2.3.1 IMHA

The first of the three diseases of relevance to this thesis, IMHA is a commonly seen disease in dogs, in which immune-mediated destruction of red blood cells occurs. The mechanism behind this is a classical type II hypersensitivity response which involves autoantibody production and antibody-mediated cytotoxicity. This can lead to either haemolysis of red cells within the vasculature, due to activation of complement and formation of the membrane attack complex, or extravascular haemolysis when the autoantibodies bind to the red cells and act as opsonins, leading to phagocytosis by macrophages located primarily in the liver and spleen. IMHA can be classed as a primary disease due to immune dysregulation of unknown aetiology, or secondary due to a wide range of triggers such as infection, neoplasia, vaccination, and various drugs and toxins. Despite advances in our understanding of the pathophysiology of IMHA in recent years, the mortality rate in this disease remains high, reported as between 30 and 70% (Goggs et al., 2015).

There are a number of studies evaluating risk factors for mortality in canine IMHA, and a few also investigating specific risk factors for thrombosis. Individual prognostic factors identified for IMHA include thrombocytopenia, hyperbilirubinemia, neutrophilia, PT and aPTT, hypoalbuminemia, hyperkalaemia, bicarbonate concentration, increased creatinine kinase (CK) and alanine aminotransferase (ALT) activity, severity of azotaemia, presence of pigmenturia, and positive autoagglutination (Carr et al., 2002; Goggs et al., 2015; Piek et al., 2008; Swann and Skelly, 2015). A number of scoring systems for disease severity have also been evaluated for prognostic utility in IMHA. Of these, the American Association of Anaesthesiologists (ASA) score is a non-specific scoring system initially developed to stratify risk for patients undergoing general anaesthesia. ASA scores are assigned as follows: Grade 1, Normal; Grade 2, Mild systemic disease; Grade 3, Severe systemic disease; Grade 4, Life-threatening systemic disease; Grade 5, Moribund patient, not expected to survive. In the largest study of IMHA patients to date, ASA score >3 had an increased odds ratio of 2.7 (95% CI 1.1-6.9) for mortality (Goggs et al., 2015). Two disease specific scores have also been developed for IMHA; the Tokyo score and the canine haemolytic anaemia objective score (CHAOS). Of these CHAOS score >3

has been found to be associated with an increased odds ratio of 4.2 times (95% CI 2.2-8.0) risk of mortality during hospitalisation (Goggs et al., 2015). CHAOS score is calculated based on patient age, rectal temperature, presence or absence of autoagglutination, and serum albumin and bilirubin levels as shown in Table 2.

Table 2: Canine haemolytic anaemia objective score (CHAOS)

Age (year)	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

1.2.3.1.1 Prevalence of thrombosis in IMHA

One of the main causes of morbidity and mortality in these patients is thromboembolism, with the prevalence of thrombosis being as high as 80% at necropsy (McManus and Craig, 2001). As such, IMHA is considered a disease with a high risk of thromboembolic complications, particularly in the first 2 weeks of therapy (deLaforcade et al., 2019). Most common are venous thrombi, with portal vein, cephalic vein, splenic vein and hepatic vein thrombosis all being reported. Venous thrombi that dislodge from their initial site of formation and embolise via the vasculature to a distant site are also an important cause of pulmonary arterial thromboembolism. Arterial thrombi can also be seen, however, with splenic, renal, cardiac, iliac and mesenteric artery infarction being described in the literature (McManus and Craig, 2001; Piek et al., 2008). Unlike risk factors for mortality, individual patient risk factors for thrombosis have not been as widely investigated in IMHA. Risk factors for thrombosis identified so far include severe thrombocytopenia <50, severe hyperbilirubinemia (>85.5umol/l), hypoalbuminemia (<26g/l), increased ALP (>60IU/l), moderate to severe leucocytosis (>28x109/l), and D-dimers over 1000ng/ml (Carr et al., 2002; McManus and Craig, 2001; Nelson and Andreasen, 2003)

In human medicine, autoimmune haemolytic anaemia (AIHA) is a very rare disease compared to in dogs, occurring at a rate of only 1-3 cases per 100,000 person-years. Just as in veterinary medicine, however, there is an increased risk of thrombosis, with thrombi occurring in approximately 1 in 5

patients with AIHA. In a recent meta-analysis, the risk in AIHA patients was around 2.6 times that of controls (Ungprasert et al., 2015).

1.2.3.1.2 Pathophysiology of thrombosis in IMHA

Of the three diseases covered in this thesis, by far the largest amount of research has been done into the underlying cause of thrombosis in IMHA. Overall, the mechanism behind the prothrombotic tendency in IMHA is most likely multifactorial. Of the three aspects of Virchows triad, abnormalities to the vasculature and haemostasis itself seem to be the main contributors, with significant interactions with inflammation. IMHA is well recognised as being a highly inflammatory state, and there are numerous proposed routes that this leads to both endothelial injury and activation of haemostasis (Kidd and Mackman, 2013). Prothrombotic mechanisms suggested to date in IMHA include increased platelet reactivity, exposure of TF intravascularly, exposure of PS on damaged erythrocytes and microparticles, release of procoagulant thromboplastin and heme from lysed RBCs, alterations in coagulation factor levels, and more generally the effects of hypoxia, release of inflammatory mediators, endothelial injury, and alterations to blood viscosity due to anaemia (Fenty et al., 2011; Hamzianpour and Chan, 2016; Kidd et al., 2015; Kidd and Mackman, 2013; Piek et al., 2011; Ridyard et al., 2010; Scott-Moncrieff et al., 2001; Ungprasert et al., 2015; Weiss and Brazzell, 2006a). There are varying strengths of evidence base behind these suggestions, which are outlined in the following section.

Primary haemostatic abnormalities

Starting with primary haemostatic abnormalities, there are a number of studies evaluating the contribution of increased platelet activation to the prothrombotic state in canine IMHA. A study by Weiss and Brazzell in 2006 found evidence supporting the idea that there is a population of circulating activated platelets in dogs with IMHA. In this study they assessed platelet P-selectin surface expression using flow cytometry at baseline in 20 normal dogs and 20 dogs with primary IMHA. They found the IMHA dogs had an 8.1-fold greater median P-selectin expression than the healthy reference dogs, however there was a degree of overlap, with only 15 of the 20 IMHA dogs having higher than reference P-selectin levels when the dogs were assessed individually. The percentage of platelets with increased P-selectin in these dogs at baseline was also very inconsistent, varying from 12.4 to 43.6%. This study suggests that some dogs with IMHA do have platelets circulating in an activated state, but also raises the question of whether differences in the proportion of circulating activated platelets may contribute to the risk of thrombosis in individual patients (Weiss and Brazzell, 2006b). It has been questioned, however, whether the increased P-selectin is specific to the immune-mediated pathology in IMHA, or whether it is also seen more generally in inflammatory conditions, not all of which are prothrombotic.

A study by Moritz et al 2005 showed a high mean P-selectin in 8/11 dogs with non-septic inflammation, and 4/9 with septic inflammation. Likewise there was an increased percentage of platelets with high P-selectin levels in 8 of 11 (73%) dogs with non-septic inflammatory disease, and 5 of 9 (56%) dogs with septic inflammatory disease. From this the authors concluded that at least 60% of dogs with inflammatory disease had circulating activated platelets (Moritz et al., 2005). A similar study in 2015 using flow cytometry also found evidence of platelet hyper-reactivity in critically ill dogs (Majoy et al., 2015). This raises the question of whether platelet activation alone in IMHA can be blamed for the increased thrombotic risk, given that many other canine inflammatory conditions don't show the same incidence of thrombotic disease that IMHA does. Additionally, some investigators using P-selectin have found conflicting results to those above. One such study found no alteration to platelet P-selectin expression overall in 14 dogs with IMHA compared to normal controls using whole blood flow cytometry. When the subset of 5 dogs with severe thrombocytopenia was compared to the normal controls however, there was a significant increase in platelet P-selectin. They also identified a strong association between thrombocytopenia and the other markers of platelet activation; platelet fibrinogen binding and platelet derived microparticles (Ridyard et al., 2010). Overall these studies using P-selectin do provide evidence that some dogs with IMHA will have increased platelet activation compared to normal dogs, but also add to the theory that different individuals with IMHA likely show different spectrums of abnormalities of haemostasis contributing to their overall risk of thrombosis.

Additional evidence for a role of platelets in the prothrombotic nature of IMHA comes from studies looking at routine platelet indices in clinical cases. In a retrospective case-control study, Zoia et al showed a significantly lower plasma mean platelet component (MPC) concentration in dogs with IMHA as compared to both healthy controls and to a population of dogs with other systemic illnesses (Zoia et al., 2018). MPC is a measure of platelet density, measured using light scatter on some automated haematology analysers. Previous work has found that MPC can be used to assess platelet activation, since the action of degranulation reduces the platelet density, and so the measured MPC (Chapman et al., 2003; Macey et al., 1999). MPC has also been found to correlate with P-selectin expression (Moritz et al., 2005). Low MPC in dogs with IMHA and those with DIC compared to healthy dogs and dogs with sepsis was also identified in a study primarily assessing intravascular TF expression in IMHA patients (Piek et al., 2011). Overall these studies show convincing evidence of increased platelet activation in dogs with IMHA. As yet, however, links between increased platelet activation and thrombosis itself have not been widely explored.

Other haemostatic abnormalities

Moving onto secondary haemostatic abnormalities, there is also a substantial body of work looking at abnormalities of the clotting cascade causing hypercoagulability in dogs with IMHA. Decreased AT, prolonged aPTT, prolonged PT and elevated D-dimers are commonly reported in dogs with IMHA, consistent with activation of coagulation and consumption of clotting factors (Scott-Moncrieff et al., 2001). In one study reduced activities of all clotting factors apart from VIII and IX was found in dogs with primary IMHA, consistent with the presence of a consumptive coagulopathy in at least some of these dogs (Piek et al., 2011). They also identified a correlation between aPTT and survival in multivariate analysis. The significance of these alterations as a cause of thrombosis rather than as a result of thrombosis however is unclear, and in many studies no consistent alterations to the clotting times have been seen. For example, in one such study, only one of 29 dogs with IMHA had a mildly prolonged PT, while 18 had mild prolongation of the aPTT (Goggs et al., 2012).

Several investigators have also looked into global hypercoagulability in IMHA using the newer viscoelastic assays TEG and rotational thromboelastometry (ROTEM), and identified a number of abnormalities. The first such retrospective study of 39 dogs diagnosed with IMHA found that 33 of them (85%) had hypercoagulable TEG traces based on calculation of a coagulation index (CI). This coagulation index was calculated from the four routinely assessed TEG variables (R, α, K and MA), and the calculation had previously been developed in dogs to give a single overall number as a representation of coagulation status. Overall 76% of the TEG values were hypercoagulable (Sinnott and Otto, 2009). More recently, using standard TEG parameters, Goggs et al also found that dogs with IMHA were significantly hypercoagulable compared to controls (Goggs et al., 2012). Due to concerns that many of the dogs in previous studies had already been started on medication at the time of sampling, some of which may itself affect coagulation, Fenty et al, assessed 11 dogs with IMHA using TEG specifically prior to administration of any immunosuppressants, anticoagulants, or transfusions. While no overall CI was calculated, again all 11 dogs in this study were deemed hypercoagulable, with a lower median K, and higher median α and median MA (Fenty et al., 2011). Some investigators have also attempted to pick apart the impact of platelet and coagulation factors on TEG trace abnormalities. In one such study, authors found that the majority (70%) of 27 dogs with IMHA had thromboelastograms consistent with hypercoagulability, and further characterised 50% of the hypercoagulable dogs as being due to platelet hyperreactivity, and 50% as due to clotting factors (Hamzianpour and Chan, 2016). Overall, although there remains debate over how to standardise viscoelastic test methodology so results of these studies are clinically applicable between different hospitals, these tests provide evidence of a variety of abnormalities of secondary haemostasis present in dogs with IMHA.

Links with inflammation

While as outlined above, there is clearly a growing body of evidence to suggest alterations to platelet activation and the clotting cascade are present in IMHA, a range of other abnormalities associated with the pro-inflammatory state have been found that may make these patients prothrombotic. The first of these is hyperfibrinogenaemia. Numerous studies have identified increased circulating fibrinogen levels in dogs with IMHA (Fenty et al., 2011; Kidd et al., 2015; Piek et al., 2011; Scott-Moncrieff et al., 2001). Fibrinogen is an acute phase protein, produced by the liver in many inflammatory conditions, but is also the basis for fibrin production by prothrombin. As such, there has been some suggestion that hyperfibrinogenaemia may in part contribute to the hypercoagulable state.

Equally, increased expression of TF, both on endothelial cells and other cell types, has been proposed as a prothrombotic trigger. In a study by Piek et al, whole blood TF gene expression has been found to be high in dogs with primary IMHA. It is of note, however, that TF expression showed no significant association with death in the survival analysis, and blood TF expression was also high in dogs with neoplasia and sepsis when compared with normal healthy controls. The authors additionally found an association between the increase in TF expression and the platelet parameters mean platelet volume (MPV), mean platelet mass (MPM) and platelet distribution width (PDW), and hypothesised that this suggests a role for platelets in the increased TF expression (Piek et al., 2011). Previous studies have found TF mRNA expression in platelets, but also in monocytes and neutrophils (Mezzano et al., 2008; Nakamura et al., 2004; Østerud, 2010) . In the human field, heme released from haemolysed red blood cells has also been found to induce expression of TF on endothelial cells, and is a potential mechanism behind the increased TF levels found in the canine IMHA studies (Setty et al., 2008).

Investigators have also found evidence of increased microparticle numbers in dogs with IMHA (Kidd et al., 2015; Ridyard et al., 2010). Microparticles are produced by blebbing of the cell membrane of multiple different cell types, including RBC, platelets, endothelial cells, and leucocytes such as monocytes. Numerous studies in humans have shown that microparticles from all these cell lines can express prothrombotic markers such as PS on their surface. This expression has been hypothesised to be how microparticles contribute to a prothrombotic state, as in particular platelet-derived microparticles have been shown to be more procoagulant than activated platelets by a factor of 50 to 100 times (Sinauridze et al., 2007). In support of this, a study by Kidd et al evaluating microparticles in dogs with IMHA found that while the absolute number of microparticles positive for PS surface expression (PS+) was not significantly higher in dogs with IMHA than control dogs, the median in vitro thrombin generation, used as a measure of procoagulant activity, associated with those microparticles was higher for the IMHA dogs than for the controls. Notably, however, this median increase in the MP

procoagulant activity in the group overall was almost entirely due to three individual IMHA dogs, with 10 of the 15 dogs having levels comparable to the controls. Notably, those 3 IMHA dogs all showed evidence of intravascular haemolysis based on marked hemoglobinemia. The authors also found higher factor Xa generation from microparticles in 4 of the 15 IMHA patients than controls, and specifically higher tissue-factor dependent FXa production in some. They did not, however, correlate these findings with clinical outcome, so whether thrombosis is more likely when MP procoagulant activity is increased is unknown (Kidd et al., 2015). Taken together, however, these findings add weight to the theory that microparticles are involved in the prothrombotic state in some, although possibly not all, dogs with IMHA. In the human field, it has also been found that microparticle levels increased in stored red blood cells used for transfusion (Rubin et al., 2010). Given that a significant proportion of dogs with IMHA will require transfusion support, it has been suggested that transfusion therapy may also be a contributor to prothrombotic tendencies in our patients. Similarly, damage to red cells caused by haemolysis leads to exposure of normally intracellular antigens to the circulation. Of particular relevance is the exposure of PS on damaged red cells (Ataga, 2009). In the same study by Kidd et al evaluating procoagulant activity associated with microparticles in dogs, mechanical haemolysis increased in vitro procoagulant activity associated with PS+ and TF+ microparticles (Kidd et al., 2015). While the cause of this was not specifically investigated, either red cell fragments expressing PS or red cell derived microparticles could explain these findings. Additionally, substances such as heme and ADP released from red cells can act as erythrocyte-derived damage-associated molecular pattern molecules (DAMPs), and by binding to pattern recognition receptors on multiple cell types including platelets, can promote both inflammation and thrombosis (Vogel and Thein, 2018).

Finally, increases in blood-borne chromatin complexes released by activated neutrophils, named neutrophil extracellular traps (NETs), have also been shown to be present in dogs with IMHA. In a prospective study assessing NETs concentrations in 35 dogs with IMHA, there was also a trend towards higher NETs levels in non-survivors than survivors, although this did not reach statistical significance (Lawson et al., 2018). Several studies in humans and rodent models have implicated NETs in the development of thrombi, in part by acting as a trigger for platelet activation, and so increases in NETs has been proposed as another contributor to the prothrombotic state in canine IMHA patients (Brill et al., 2012).

Overall, the mechanism behind the prothrombotic tendency in dogs with IMHA is likely multifactorial, and may differ in severity between individual patients. While better understanding of the molecular mechanisms behind the thrombotic tendency in IMHA may identify targets for therapeutic intervention, applying this information clinically will remain difficult without an easily accessible test or biomarker for overall thrombotic risk.

1.2.3.2 PLN

The second disease of interest in this thesis, PLN, is also relatively commonly seen in canine patients. Damage to the renal glomeruli from various disease processes causes disruption to the normal filtration barrier, resulting in increased protein loss in the urine (Littman, 2011). Lower molecular weight proteins such as albumin and AT are most easily lost, although the overall charge on the molecules also affects how easily they pass through the glomerular membrane. Affected dogs are commonly hypoalbuminaemic, and can have associated clinical signs of weight loss, muscle wasting, azotaemia, peripheral oedema and ascites. The occurrence of the combination of hypoalbuminemia, marked proteinuria, hyperlipidaemia and fluid accumulation such as ascites or peripheral oedema is termed nephrotic syndrome, and is a rare but severe complication of PLN (Klosterman et al., 2011; Klosterman and Pressler, 2011; Littman, 2011).

Similarly to IMHA, PLN can be secondary to a variety of disease processes, such as neoplasia and inflammatory diseases, and can be a primary condition in its own right. Primary PLN can also be congenital, or an acquired abnormality. In veterinary medicine, a diagnosis of PLN is based on detection of significant proteinuria, and establishing it is of renal origin by exclusion of pre- and post-renal causes (Littman, 2011). Pre-renal causes of proteinuria involve increased delivery of low molecular weight proteins to the glomeruli, and include conditions causing haemoglobinaemia, myoglobinaemia, and with increased levels of immunoglobulin light chains in certain cases of lymphoma and multiple myeloma. Post-renal causes of proteinuria include lower urinary tract diseases such as urinary tract infection, urolithiasis, or neoplasia of the lower urinary or genital tract (Lees et al., 2005). Once proteinuria has been determined to be renal in origin, PLN can then be further investigated by means of renal biopsy to establish the histopathological cause, although this is not always pursued clinically due to the risks of the biopsy procedure. Histopathological changes underlying PLN can include congenital glomerular malformations, acquired immune-mediated glomerulonephritis, reactive amyloidosis, and glomerulosclerosis (Littman et al., 2013). In many cases further screening to identify underlying conditions behind a secondary PLN, in particular in the case of immune-mediated glomerulonephritis, is also performed (Littman, 2011; Littman et al., 2013).

1.2.3.2.1 Prevalence of thrombosis in PLN

Thrombotic disease is a well-recognised potential complication of PLN, and in one post-mortem study was seen in 25% of cases (Cook and Cowgill, 1996). Other more recent prospective cross-sectional studies have reported a similar prevalence of thrombosis of between 6.6% and 27% (Donahue et al., 2011; Lennon et al., 2013; White et al., 2016). As with IMHA, both venous thrombi and arterial thrombi have been reported. In human medicine, patients with nephrotic syndrome, a severe form of PLN, also

have an increased risk of thrombotic disease, with thrombi occurring in between 20 and 37% of adult patients (Singhal and Brimble, 2006)(Eneman et al., 2016).

1.2.3.2.2 Pathophysiology of thrombosis in PLN

As with IMHA the mechanism behind the thrombotic tendency in PLN is not fully understood, however classically has been largely attributed to abnormalities of secondary haemostasis due to AT deficiency. In an early case report of three dogs with PLN, AT levels were reduced to between 74 and 32% of normal, hypothesised to be due to increased urinary loss of this small protein. Likewise in human medicine, AT deficiency is seen in between 40 and 80% of patients, and some but not all studies have shown an association with deep vein and pulmonary thrombosis (Singhal and Brimble, 2006). In recent years there has been a renewed interest in the pathophysiology of thrombosis in PLN, in particular in using viscoelastic testing to investigate additional abnormalities of secondary haemostasis alongside acquired AT deficiency (Donahue et al., 2011; Lennon et al., 2013; White et al., 2016). As yet limited work has been done in the veterinary field looking into any role the endothelium or platelets may play in thrombosis in PLN. In people however, evidence for additional mechanisms contributing the prothrombotic state has been found. These include endothelial cell injury, loss of other small anticoagulant proteins such as protein C alongside AT, and of particular interest for this thesis, increased platelet activation (Eneman et al., 2016; Singhal and Brimble, 2006).

A recent study looked at TEG traces in dogs with PLN, and concurrently assessed antiplasmin, AT, D-dimers, Factor VIII, fibrinogen, plasminogen, protein c and vWF levels. Of the 11 dogs with PLN, all had hypercoagulable TEG traces, with shorter R and K times and larger α, MA and CI values compared with normal controls. They were also found to have a significantly shorter PT, lower levels of AT, and higher levels of protein C and fibrinogen. However the same changes to the TEG, and AT and fibrinogen were seen in dogs with nonprotein-losing renal failure, raising the question of whether these changes alone are enough to explain the prothrombotic tendency seen in PLN (Donahue et al., 2011). Similarly, a study using kaolin activated TEG found 27 of 28 dogs with PLN to be hypercoagulable, having lower K and higher α and MA values, with no correlation between these values and the AT activity. They also found no significant difference in the AT activity between the 4 dogs with PLN with concurrent documented thrombosis, and the remaining 24 dogs without, however the number of dogs with thrombi may have been too small to detect a clinically significant difference. Unlike the previous study, this one included both hypoalbuminaemic and normoalbuminaemic dogs with PLN, and found that both groups showed the same characteristics of hypercoagulability (Lennon et al., 2013). More recently, White et al similarly found no association between hypoalbuminemia and hypercoagulability as assessed by TF activated TEG in a larger population of dogs with PLN. They also found no correlation

between hypercoagulability on TEG and plasma AT activity. In this study the incidence of hypercoagulability as assessed by TEG was 89%, with an incidence of thrombosis of 6.6% (White et al., 2016). Overall while multiple TEG abnormalities have been described, which of these are most important in predicting thrombotic risk is unknown.

In the human field, while the alterations to the clotting cascade described above are widely described as the main abnormality driving thrombosis in patients with nephrotic syndrome, a number of different abnormalities in platelet function have also been discovered, and likely also contribute (Singhal and Brimble, 2006)(Eneman et al., 2016). These include evidence of increased platelet activation as shown in by increased in vitro platelet aggregation in response to various agonists, increased release of β-thromboglobulin and platelet factor 4, plus increased platelet surface expression of P-selectin and glycoprotein 53. While the exact mechanisms that drive this increased platelet activation are as yet unclear, associations with hypoalbuminemia, hypercholesterolaemia, and hyperfibrinogenaemia have all been described (Eneman et al., 2016). The degree to which these platelet alterations contribute to the risk of thrombosis is also unclear. In contrast to the volume of data available for humans, as yet in the veterinary field only very limited investigation of platelet activation in PLN has been performed. The first veterinary study investigating the role of platelets in PLN has suggested that hypoalbuminemia may be the main contributor to the increased thrombotic risk, via increased free plasma arachidonic acid causing increased platelet reactivity. It is worth noting, however, that although platelet hypersensitivity was found in two dogs using light aggregometry, both were additionally found to be infected with heartworm (*Dirofilaria immitis*)(Green et al., 1985). The adult worms of Dirofilaria live within the pulmonary vasculature, and have been found to induce coagulation dysfunction, although the mechanisms behind this are not fully elucidated (González-Miguel et al., 2012). As such it is possible Dirofilaria rather than the PLE was behind the increased platelet activation in this study. Although there have been more recent studies evaluating the impact of uraemia on platelet function in dogs with CKD, which found evidence of increased platelet activation by means of surface P-selectin expression measurement, no investigators have looked further into the role of platelet function in dogs with PLN (Dudley et al., 2017). Other suggested contributors to the hypercoagulable state in PLN are increased activity of clotting factors V, VII, VIII and X, increased vWF concentrations, and reduced plasminogen concentration. Overall, however, while PLN is accepted to be a prothrombotic condition, relatively little is known about the underlying prothrombotic mechanisms in dogs with PLN compared to those with IMHA.

1.2.3.3 PLE

The final prothrombotic condition of interest, PLE, is a syndrome that can be seen with a variety of severe gastrointestinal disorders. It is characterised by increased protein loss through the intestinal tract, leading to hypoalbuminemia. The underlying mechanism behind PLE can vary from case to case, but always involves sufficient damage to the intestinal tract such that intestinal protein loss exceeds systemic protein synthesis. Diseases that can underlie PLE in dogs include chronic enteritis, intestinal lymphangiectasia and intestinal lymphoma, however inflammatory bowel diseases (IBD) are the most commonly seen cause. Protein losing enteropathy can present solely with gastrointestinal signs associated with the underlying intestinal pathology, but as with PLN can also present with signs of ascites or oedema due to hypoalbuminemia. In people PLE is more commonly caused by lymphangiectasia, rather than by IBD. However human IBD is similarly associated with thrombotic complications, and so is the closest disease for pathophysiological comparison (Craven and Washabau, 2019).

As with IMHA, there have been a number of studies looking for prognostic factors in canine patients with both PLE and IBD. Two disease severity scoring systems based on clinical signs and clinicopathological findings have been developed for IBD; the CIBDAI (canine IBD activity index) and CCECAI (canine chronic enteropathy clinical activity index) (Allenspach et al., 2007; Jergens, 2004). Notably multiple studies have identified hypoalbuminemia as a negative prognostic indicator in both IBD and in PLE specifically (Allenspach et al., 2007; Craven and Washabau, 2019; Gianella et al., 2017). Survival times for dogs with PLE are very variable, from a matter of weeks to several years. Eventual disease-associated death has been found to occur in around half of patients (Craven and Washabau, 2019).

1.2.3.3.1 Prevalence of thrombosis in PLE

While thrombotic disease is a recognised risk in canine patients with protein losing enteropathy, the prevalence in dogs appears much lower than in either IMHA or PLN. There are, however, very few veterinary papers assessing the prevalence specifically, which has been reported as anywhere between 0 and 15% (Craven and Washabau, 2019). In one retrospective study of 92 dogs with PLE looking at potential prognostic factors, no mention was made of any occurrence of thrombosis. Fifty six of the 92 dogs in the study died over the 5 year study period, but reasons for death were not reported (Nakashima et al., 2015). In another study specifically investigating the prevelance of hypercoagulability in dogs with PLE using TEG, two of the 15 dogs (13%) had suspected thromboembolism (Goodwin et al., 2011). Equally, in a recent case series, 8 dogs with PLE and thromboembolism were seen across 3 referral hospitals over 3 years. For the one institute that an

overall incidence of PLE cases was available, this gave an incidence of thromboembolism in PLE cases of 7.1%. Of those 8 cases, six had PTE, two had splenic vein thrombosis and one had aortic thromboembolism (Jacinto et al., 2017).

In humans, IBD patients have an approximately 2-fold increased risk of thromboembolism compared to healthy controls and patients with diseases other than IBD, with an incidence of thrombosis within the IBD population of approximately 1 in 100 (Grainge et al., 2010; Yuhara et al., 2013). Human patients are predominantly at risk of PTE and venous thromboembolism of the extremities. It has also been found that the risk of thrombosis correlates with disease activity, with patients having active flare-ups of disease at highest risk of thrombosis, with a relative risk in one study approximately 8 times that of controls (Grainge et al., 2010; Scoville et al., 2014).

1.2.3.3.2 Pathophysiology of thrombosis in PLE

As thromboembolism is less common in canine PLE compared to IMHA or PLN, the mechanism of hypercoagulability in this disease has been less extensively investigated. As with PLN, abnormalities of secondary haemostasis have classically been understood to be the main mechanism. This is predominantly due to reduced levels of coagulation factors, principally AT, secondary to enteric loss caused by the underlying intestinal pathology (Craven and Washabau, 2019; Goodwin et al., 2011). However again as with PLN, newer work in the human field is starting to suggest the role of inflammation, endothelial injury, and platelet activation may have been underappreciated contributors to thrombosis in PLE (Danese et al., 2004; Purnak and Yuksel, 2015; Senchenkova et al., 2015).

In the only prospective veterinary study to date evaluating mechanisms of hypercoagulability, the investigators assessed TEG traces, PT, aPTT, AT activity, fibrinogen and D-dimers in a population of 15 dogs with PLE. Compared with control dogs, all 15 PLE dogs had hypercoagulable TEG traces, with significantly decreased R, decreased K, increased α, and increased MA. The range of AT activity seen in the PLE dogs ranged from 46 to 121%, with 9 dogs having AT levels below the reference range of 65-145%. The median AT from the group was borderline low, at 65%. The median fibrinogen concentration was moderately increased, while D-dimers were either normal or equivocal. PT and aPTT were normal in all but one of 9 dogs in which they were checked, and the only abnormal result was a mild prolongation of aPTT. There were no statistically significant associations detected between any of the TEG parameters and the other coagulation parameters (Goodwin et al., 2011). While overall this study confirmed that there is evidence of hypercoagulability in dogs with PLE, as with other prothrombotic diseases, the mechanism and degree of abnormality in individual affected dogs varies. In particular, the finding that some but not all of the dogs had reduced AT activity suggests that while

this may in part be responsible for the hypercoagulability seen, as was historically hypothesised, it is unlikely to be the only underlying mechanism. This is in agreement with a case series of 8 dogs with thromboembolism, in which hypoantithrombinaemia was only found in one of the two cases in which it was measured (Jacinto et al., 2017). Additional factors that have been suggested as contributors to the prothrombotic risk in canine PLE patients include hyperfibrinogenaemia, hyperhomocysteinemia, possibly in turn linked to cobalamin deficiency, and hypomagnesemia (Goodwin et al., 2011; Jacinto et al., 2017).

In people, researchers have identified a number of abnormalities in coagulation and platelet activation that may contribute to the increased risk of thrombosis in IBD patients. These include increased levels of certain coagulation factors, increased circulating fibrinogen concentrations, decreased AT levels and decreases in inhibitors of coagulation such as protein C, protein S, and TFPI. Evidence of increased platelet activation associated with inflammation includes increases in the surface activation markers P-selectin, GP53, and CD40, alongside increased plasma levels of thromboxanes and β-thromboglobulin (Purnak and Yuksel, 2015). A large number of environmental risk factors such as smoking, recent immobilisation and central catheter placement have also been associated with increased risk of thrombosis in IBD patients. Overall the pathogenesis of clots in these patients is not fully understood but is considered to be multifactorial; the situation is likely to be similar in our canine patients. To date, however, veterinary research into thrombosis in PLE has focused on secondary haemostatic abnormalities, and not on the roles of the endothelium and of platelets.

1.3 Prothrombotic states in individual patients

As outlined above, IMHA, PLN and PLE are all clearly associated with thrombosis at the population level, with a range of different predisposing factors for thrombosis proposed. However, within a population of diseased animals, different individuals with have different severities of change to each of the pathophysiological mechanisms that can lead to thrombosis. Thus each individual animal will have a different cumulative overall risk of a clot forming. When these aspects of haemostasis are only mildly abnormal, the individual may not have a clinically relevant increased risk of clot formation. However when multiple aspects are severely deranged, the animal may be at very high likelihood of forming a clot. This risk is also likely to change over time, affected by factors such as therapeutics and diagnostic investigations alongside the natural progression of the underlying predisposing disease itself. At times at which the individual is at high risk of thrombosis, they can be thought of as not only having a prothrombotic disease, but also as actively being in a prothrombotic state. In an ideal world we would be able to identify when patients are in a significant enough prothrombotic state that they

are at imminent risk of thrombus formation. This would potentially allow us to target antithrombotic therapy to those patients who are most at risk, with the aims of both reducing the incidence of thrombosis in the highest risk patients, and potentially sparing the lowest risk patients unnecessary antithrombotic therapy. This could in principle be achieved in three ways; firstly by individually assessing all the aspects of haemostasis and inflammation in each patient, to give a cumulative overview of the abnormalities present, secondly by identifying which single abnormality correlates most closely with the incidence of thrombosis in the specific disease and just evaluating that parameter, or thirdly by identifying a biomarker of overall thrombotic risk. At present, while a number of tests show some promise for utility in detecting a prothrombotic state in individual patients, they all have significant limitations.

1.3.1 Detecting a prothrombotic state

1.3.1.1 Platelet activation testing

Given increased platelet activation has been documented experimentally in a number of prothrombotic diseases, investigators have looked into various ways of measuring platelet activation as potential markers of a prothrombotic state in individuals. At present few of these tests are clinically available, although some are starting to become accessible in larger referral hospitals. Broadly speaking there are two approaches to assessing platelet activation; firstly using platelet function assays that directly assess the clotting potential or reactivity of platelets in vitro in response to a range of agonists, and secondly by assessing markers of platelet activation that are either expressed on the platelet surface or secreted when platelets are activated.

There are a number of methods available for platelet function testing, the majority of which are limited in availability to large specialist haematology laboratories, although in recent years attempts have been made to develop smaller bench-top analysers. While more accessible, and much less complex to run, these bench-top analysers tend to be far more limited in the information they can provide. Classically platelet function testing has been by light transmission aggregometry (LTA). This still represents the gold standard for platelet function testing in human medicine and is widely used in reference laboratories. LTA requires platelet rich plasma, generated by centrifugation of anticoagulated whole blood, and measures platelet aggregation in response to addition of a platelet agonist, via analysis of light transmission through the sample as aggregation occurs. The agonist chosen depends on the specific reason for analysis (for example diagnosis of thrombocytopathia, or for therapeutic drug monitoring). Due to the specialised equipment and training required for this test, LTA is confined to large human medical reference laboratories, and at present is not available at veterinary reference laboratories within the UK. As such Its use in veterinary medicine is largely

confined to research studies. A variant on LTA is whole blood impedance aggregometry (WBA). One of the advantages of this method is that it uses anticoagulated whole blood, so both requires less sample processing than LTA, and has been argued to evaluate coagulation in a more physiological setting than in platelet rich plasma. WBA works by measuring the change in resistance between two electrodes as platelets aggregate between them. Again, a range of different agonists can be used to induce platelet aggregation depending on the reason for testing. Like LTA, WBA cannot be used in a clinic point-of-care setting, however in human medicine it can be used in a near-patient setting, primarily in large hospital laboratories. As such, while several important studies assessing platelet function in veterinary medicine have used this technique, its use is confined to a research setting.

Because of the complex technical requirements for LTA and WBA, researchers have tried to develop similar but simpler assays. These include the Multiplate analyser, VerifyNOW analyser, the Plateletworks system, vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay, and the Platelet Function Analyser-100 (PFA-100) and Platelet Function Analyser-200 (PFA-200)(Saati et al., 2018). These are all based on similar principles of measuring changes in sample platelet number, light transmission, or electrical impedance following addition of platelet agonists, to assess how easily the platelets within a sample aggregate. Of these, only the PFA-100 and PFA-200 are true bench-top analysers. A few veterinary studies have used the PFA to assess platelet reactivity in patients with prothrombotic conditions, however no evidence of hyper-reactivity was seen using this tool in dogs with neoplasia, hyperadrenocorticism and cardiac disease (Clancey et al., 2009; Eberle and Mischke, 2010; Jeffery et al., 2016; Kol et al., 2013) . While some studies using the other laboratory-based assays have been performed in veterinary medicine, they are only just starting to be available as day-to-day tools in larger referral practices.

In routine veterinary practice, the only readily available potential marker of platelet activation is the MPC, which can be measured on automated haematology analysers readily available to veterinary practices (Macey et al., 1999). Recently there has been a renewed interest in MPC as a possible marker of platelet activation and prothrombotic risk in various situations, including canine IMHA, DIC, neoplasia, sepsis and certain parasitic infections (Goddard et al., 2015; Piek et al., 2011; Smith et al., 2014; Zoia et al., 2018) . MPC has been shown to be a possible prognostic indicator in IMHA, although as yet no studies have looked specifically into its utility for predicting thrombotic risk (Zoia et al., 2018).

The second approach to evaluating the degree of platelet reactivity is to measure various markers of activation. These markers can be substances released from platelet granules on activation, substances synthesised and excreted only when platelets are activated, or molecules expressed on the platelet surface in greater numbers following activation. The most commonly used measures are P-selectin

and metabolites of thromboxane. P-selectin levels are most commonly measured by flow cytometry. This technique uses a variety of antibodies and dyes to differentiate between blood components such as leucocytes and platelets, and to assess the level of a wide range of different proteins expressed on cell surfaces. In addition to assessment of baseline levels, platelet surface markers including P-selectin can be measured following addition of agonists such as ADP or Phorbol myristate acetate (PMA), to assess the change in surface protein expression as a marker of platelet reactivity. Flow cytometry can also be used to evaluate for other blood components such as platelet-leucocyte aggregates and microparticles, which have been suggested in some studies to be more sensitive and reliable than surface proteins such as P-selectin as markers of platelet activation (Wills et al., 2006). An additional limitation of P-selectin specifically is that it is both rapidly shed from the platelet membrane surface following activation and is also involved in leucocyte-platelet binding and aggregation. The latter feature means some surface P-selectin on activated platelets in samples from clinical patients could already be pre-bound by circulating leucocytes, meaning it will not be free for binding and detection by the immunofluorescent antibody used for flow cytometry. As such there is some debate over whether P-selectin is the best surface marker of platelet activation, or whether other additional or alternative markers should be explored (Wills et al., 2006). While at present flow cytometry measurement of platelet surface markers is a research tool, and not clinically available, flow cytometry itself can already be used clinically for investigating surface molecule expression of circulating leucocytes, so in theory a similar service could be established for platelets, should studies show clinical utility.

Finally, measurement of metabolites of activated platelets has been investigated as a potential marker of prothrombotic risk. The predominant metabolic pathway evaluated for this purpose is the thromboxane A2 pathway. Thromboxane A2 is produced by the enzyme cyclooxygenase 1 (COX-1) in activated platelets and then released into the circulation. It is a potent platelet agonist, recruiting and activating further platelets by binding to surface thromboxane receptors, and so acts as a major positive feedback loop for platelet activation. This pathway has been used to evaluate platelet activation in several ways. Firstly, direct measurement of levels of thromboxane and its downstream metabolites in plasma and urine have been investigated. Secondly, expression levels of the COX enzyme itself within platelets can be measured, again by monoclonal antibody labelling and detection using flow cytometry. These approaches have been used in a number of veterinary and human studies assessing the impact of the COX-inhibitor drug aspirin (Dudley et al., 2013). To date, however, very little work has been done in veterinary patients evaluating the potential of thromboxane metabolites as markers of platelet activation, or as biomarkers for thrombotic risk. As the major focus of this thesis,

thromboxane metabolite measurement and its possible roles in thromboembolic disease is expanded upon in a subsequent section below.

1.3.1.2 Viscoelastic testing

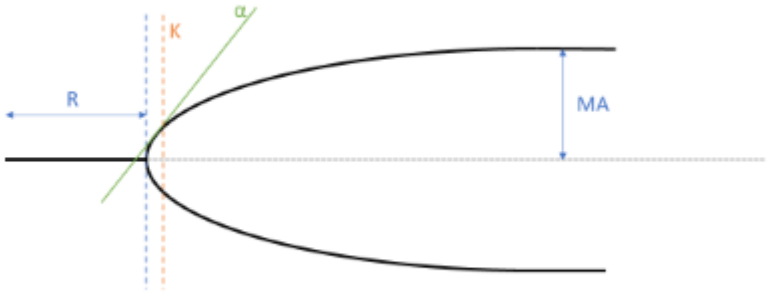
The assays that have received the most attention recently for their potential utility to identify a prothrombotic stage are the viscoelastic assays of global coagulation; Thromboelastography (TEG) and ROTEM. These viscoelastic techniques aim to provide data on all aspects of haemostasis, from the time to clot initiation to measures of fibrinolysis. As such these methods are said to give a measure of 'global' haemostasis, being able to assess for both hypercoagulability and hypocoagulability as well as platelet factors. It has also been suggested it may be better for predicting the overall risk of thrombosis in patients with multifactorial causes for being prothrombotic, as it allows the platelet and clotting factor components of coagulation to interact, rather than assessing them independently as with classical coagulation tests. This is felt to represent a more global view of clotting, including more of the complex interactions between clotting and cellular surfaces explored by the cell-based model of haemostasis. The drawback, however, is that they are still ex-vivo tests, and so cannot take into account the role of the endothelium, or of blood stasis (Donahue and Otto, 2005).

Practically, TEG involves incubating anticoagulated whole blood in a sample chamber with an oscillating pin suspended in the centre. The basic principle is that clot formation in the whole blood sample slows and then stops the vibration of a central pin, with the magnitude of the vibrations over time being recorded and produced as an output trace. Various measures can be taken from this trace, including the time for clotting to start, the speed at which a clot forms, the overall strength of that clot, and the speed with which the clot is then broken down. Common measurements used are the lag time before the clot starts to form ('reaction time 'R'), the rate at which clotting occurs (K), the clot formation angle (α), the maximal amplitude of the trace (MA), which represents clot strength, and the rate of amplitude reduction as a measure of fibrinolysis (Figure 2). These different aspects of the trace are affected to varying extents by the different aspects of the clotting cascade and platelet function, so can be used to a degree to help determine the origin of hyper- or hypo-coagulability detected. For example, the reaction time R has been found to be principally a measure of the speed of the reactions of the intrinsic pathway, meaning it is affected by alterations to the concentrations of factors VIII, IX, XI and XII. The rate of clotting to a predefined clot strength, K, is affected by both platelet numbers and function, alongside the levels of fibrinogen, thrombin, factor VII, and also by the haematocrit. The strength of the clot formed, represented by MA, is also affected by platelet count, function, haematocrit, fibrinogen and thrombin, but also by factor XIII.

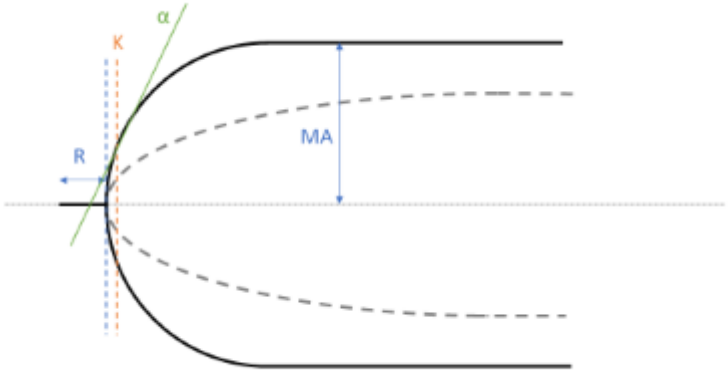
As discussed in more detail for IMHA, PLE and PLN specifically below, viscoelastic tests have been used to identify a hypercoagulable state in a number of canine diseases, including hyperadrenocorticism , IMHA, PLN, neoplasia, and in critically ill dogs with a range of conditions (Fenty et al., 2011; Goggs et al., 2012; Hamzianpour and Chan, 2016; Pace et al., 2013; Sinnott and Otto, 2009). These studies use a range of parameters generated by the TEG to define 'hypercoagulability', and as of yet there is no consensus as to which are the most useful or appropriate. In addition, none of these studies correlate the finding of a hypercoagulable TEG tracing with the occurrence rate of thrombosis in the population involved. As previously discussed, the only study to attempt this found no association between any of the TEG parameters and the incidence of thrombosis in the dogs at necropsy up to 7 days later (Thawley et al., 2016). In human medicine a recent systematic review found some evidence that TEG could be used to predict the risk of thromboembolism in post-operative surgical patients, however there was a large degree of variability in the sensitivity (0 – 100%) and specificity (62-92%)(Dai et al., 2009). As in the veterinary world, there were also different definitions of what constituted a hypercoagulable tracing used in the different individual studies reviewed. At present, viscoelastic tests may represent a promising avenue for further research into predicting thrombotic risk in veterinary patients, however as mentioned previously, comparison between centres is still limited by inter-machine variability.

Figure 2: TEG trace examples

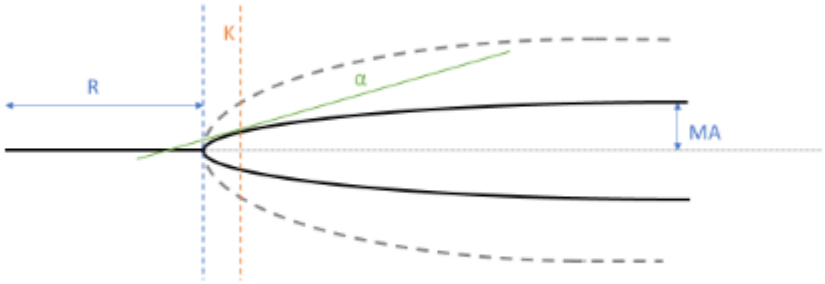
A.



B.



C.



Legend: Figure 2A: A normal TEG trace. Figure 2B: A TEG trace with changes consistent with hypercoagulability including shortened R, shortened K, increased α and increased MA. Figure 2C: A TEG trace with changes consistent with hypocoagulability including prolonged R and K, and reduced α and MA.

1.3.1.3 D-dimers

As discussed above the specificity of D-dimers for the presence of a detectable thrombus tends to be low, with increased D-dimers being seen in a variety of conditions. However it has been speculated that this may be due to D-dimers actually being very sensitive for a prothrombotic state, in which thrombi are being partially formed, but fibrinolysis and coagulation are occurring in balance, so thrombi of a clinically relevant size are never formed (Jeffery et al., 2016). Supporting this idea are a number of population-level human studies in which elevated D-dimers are associated with an increased long-term risk of subsequent venous thrombosis. One such study found an overall increased adjusted hazard ratio of 3.2 for venous thromboembolism (either DVT or PTE) in the patients in the initial highest quartile of D-dimer values (0.51- 20 ug/ml) compared to the lowest quartile (0.01-0.14 ug/ml) in over 12000 participants over a 17-year study period (Folsom et al., 2015). Whether this relationship seen at the population level also applies to D-dimers as a predictor of thrombotic risk in an individual human patient is less clear. In veterinary medicine, D-dimers have to date only been evaluated for detecting existing thrombosis; to the authors knowledge there are no longitudinal studies looking at future risk of thrombosis based on D-dimer levels, either in diseased or healthy canine populations. Future studies in this area may help to evaluate D-dimers as markers of prothrombotic tendencies, although differences in performance and agreement between different canine D-dimer assays are a significant obstacle that need to be addressed to make this feasible (Jeffery et al., 2016).

1.3.1.4 Classical secondary haemostatic tests

Of the classical tests of coagulation, while any association with the presence of a gross thrombus is doubtful, PT and aPTT have attracted recent attention as possible indicators of hypercoagulability. Historically in veterinary medicine, while prolongation of the clotting times PT and aPTT have been associated with hypocoagulability and bleeding tendencies, shortening of both the PT and aPTT have been thought to be of no clinical significance. In human medicine, however, a shortened aPTT has been associated with an increased risk of long-term venous thromboembolism. In a recent study of over 13,000 patients over 13 years, patients with a starting aPTT in the lowest quartile had a 2.4x higher risk of venous thrombosis over those with aPTT in the highest quartile, although the incidence of venous thrombosis in the population overall was low (Zakai et al., 2008). A relationship between degree of shortening of the aPTT and the risk of venous thrombus has also been identified, with the adjusted odds ratio for VTE increasing with decreasing aPTT ratio (Tripodi et al., 2004).

In the veterinary field, a recent retrospective study found that dogs with shortened PT or aPTT had a significantly higher overall incidence of thrombosis compared to controls (70% vs 39%). Suspicion of

PTE specifically was also seen more commonly in cases (61%) as compared with controls (9%), and of all the dogs with suspected PTE, 88% had a shortened PT or aPTT (Song et al., 2016). Whether the shortened PT and aPTT in this study reflect an underlying hypercoagulable state, or the presence of a thrombus itself and active consumption of clotting factors is debatable. As this is the only study to date assessing the relevance of shortened PT and aPTT in dogs, further studies evaluating this possible link with hypercoagulability are warranted before these clotting times can be considered useful in helping detect a prothrombotic state in individual patients.

1.3.1.5 Other Novel markers

Given the limitations in our currently clinically available tests to predict which patients are prothrombotic, there is unsurprisingly a lot of ongoing research in this area in both human medicine and veterinary medicine, and a number of novel potential markers have been identified for future use. In humans, serum levels of antiphospholipid antibodies have been shown to be predictive of venous thrombosis risk in certain situations (Lijfering et al., 2010). Canine antiphospholipid antibodies can be measured by ELISA, and so could relatively easily be offered as a clinically available diagnostic test by laboratories already offering other canine ELISA based tests. However an initial study looking at levels in healthy dogs, as compared with sick dogs without thrombosis, and dogs with either IMHA, spontaneous thrombosis, or hyperadrenocorticism showed no evidence of a difference in antibody prevalence between the groups to suggest use as a predictor of thrombotic risk in dogs (Miller et al., 2012). As such at present antiphospholipid antibodies do not appear to be a clinically useful measure of individual patient's prothrombotic state.

Circulating microparticle levels have also recently been identified as potentially useful markers of prothrombotic tendency, both in the human and veterinary fields. Microparticles can be measured in two ways; either by flow cytometry which uses antibodies to quantify the number of microparticles of different cells of origin in a sample, or by functional assays, which may better assess the physiological relevance of the detected microparticles but does not quantify the number present nor identify their cell of origin (Jeffery et al., 2016). In human medicine, several prospective studies, particularly in patients with neoplasia, suggest blood levels of tissue-factor bearing microparticles may predict risk of venous thrombosis. Whether microparticles have the same predictive use in human patients that develop venous thrombi without an underlying neoplasia is still unclear however. In a recent study, microparticle levels measured by flow cytometry were compared between patients with newly diagnosed venous thrombi and healthy controls. They showed a significantly higher median microparticle level in the patients with thrombi, and a linear association between the level of microparticles and the risk of thrombi, although there was a significant overlap between the level of

microparticles seen in the two populations (Bucciarelli et al., 2012). Microparticles have also been investigated for association with thrombosis in a number of veterinary studies, as outlined for IMHA in section 1.2.3.1.2 above, but have not to date been evaluated as a predictor of future thrombosis in individual patients.

1.3.2 Detecting a prothrombotic state in patients with IMHA

Of the tests of platelet activation outlined above, only platelet surface markers, microparticle levels, and MPC have to date been evaluated in dogs with IMHA. Platelet surface P-selectin expression has been found to be increased in dogs with IMHA compared to healthy controls, but interestingly the degree of increase is not consistent across the IMHA dogs. In one study 15 of the total 20 IMHA dogs had higher than reference P-selectin levels when the dogs were assessed individually. Additionally, the percentage of individual platelets within a single dog showing increased P-selectin was very inconsistent, varying from 12.4 to 43.6%. The authors further investigated whether there was any correlation between the P-selectin expression and the clinical occurrence of thromboembolism. Seven of the dogs had clinical signs suggestive of thromboembolism, but there was no difference in the mean P-selectin expression between those dogs with suspected thromboembolism and those without (Weiss and Brazzell, 2006b). As such it is currently unclear whether platelet surface marker expression is likely to be a useful tool clinically for predicting thrombotic risk in individual veterinary patients. Logically, it seems likely that a panel of surface markers beyond just P-selectin expression may give more information than a single marker, as is the situation for flow cytometry analysis of leucocyte surface molecules. At present, the only clinically available measure of platelet activation is platelet MPC. Only one study has evaluated MPC in clinical IMHA cases so far, but the authors found a significant association between MPC and outcome, with a decrease in MPC of 1 unit associated with a 16% increased risk of death. They did not, however, evaluate for any association with thrombosis itself (Zoia et al., 2018). As such, it remains to be seen whether MPC may have future utility in this role.

The viscoelastic test TEG is probably the most clinically useful test of hypercoagulability in dogs with IMHA currently, although access to the test is currently limited to certain large veterinary hospitals. In the existing studies evaluating TEG abnormalities in IMHA dogs, some associations with survival in individual dogs have been found. Interestingly, whether relatively hypercoagulable, hypocoagulable, or normocoagulable traces are linked with worse prognosis is variable between studies. In a study using a calculated coagulation index (CI), authors found a normal CI was significantly associated with decreased survival compared with having a hypercoagulable CI. None of the 6 dogs with normal CI survived, while 56% of the hypercoagulable dogs survived. Overall 76% of the individual TEG

parameters were hypercoagulable, however there was no statistical association between any of these 4 TEG parameters and survival status (Sinnott and Otto, 2009). In contrast, more recently, using standard TEG parameters, Goggs et al found that dogs with IMHA were significantly hypercoagulable compared to controls, but also found a significant association between increased MA and survival, with relative hypocoagulability at admission being a negative prognostic indicator. It was hypothesised that this relative hypocoagulability was consistent with a consumptive coagulopathy, so explaining the link to a poorer prognosis. They also tracked the TEG changes over the course of treatment in these dogs, and found that over the first 5 days the R and K values significantly increased, while the MA value decreased, overall consistent with worsening hypercoagulability (Goggs et al., 2012). Notably none of these studies assessed for associations between TEG variables and the subsequent incidence of thrombosis in these patients, so at present while we know there are potential associations with survival, we do not yet know whether TEG can be used to help quantify which patients are in a prothrombotic state, and so at increased risk of thrombosis.

Equally, of the other research methods that have identified abnormalities that could contribute to thrombosis in IMHA, including microparticle analysis, intravascular TF expression, circulating NETs levels, and PS expression, none has as yet been evaluated for associations with the occurrence of thrombosis in clinical patients. Additionally, at present none of these methods are easily accessible to clinicians in practice, even in academic referral institutions. Overall while there is a strong body of evidence that numerous different abnormalities can contribute to the prothrombotic tendency in IMHA, there is limited information on how we can test for thrombotic risk and the presence or absence of a prothrombotic state in individual patients within the clinic.

1.3.3 Detecting a prothrombotic state in patients with *PLN*

As outlined above, the majority of work looking at underlying causes for thrombosis in canine PLN patients has focused on the role of AT deficiency. While a number of studies have identified low AT levels in dogs with PLN, fewer have sought to assess an association between AT levels and the subsequent incidence of thrombosis in individual dogs. The one study to attempt to do this found no significant difference in AT levels between the 4 PLN dogs with concurrent thrombosis, and the other 24 PLN dogs in the study (Lennon et al., 2013). The authors did comment, however, that the number of dogs with thrombi may have been too small to detect a clinically significant difference. Likewise, where researchers have investigated TEG abnormalities in dogs with PLN, no attempt to correlate the findings with the occurrence of thrombosis have been made. Authors of one study noted that the incidence of hypercoagulability as assessed by TEG was 89%, while the prevalence of thrombosis in the group was 6.6%, but no further analysis was performed (White et al., 2016). Overall while we are

starting to identify additional prothrombotic abnormalities present in dogs with PLN, we are a long way from establishing which are most clinically relevant, and which may have potential to be used as biomarkers in individual patients. Additionally, very little is currently known about whether significant platelet activation is present in these patients.

1.3.4 Detecting a prothrombotic state in patients with *PLE*

Of the three diseases, least is known about the overall mechanisms that make dogs with PLE prothrombotic, likely because of the lower prevalence of thrombosis compared to IMHA and PLN. In the single study evaluating haemostatic abnormalities in dogs with PLE, all 15 dogs were prothrombotic based on TEG, but no ongoing follow-up to document the incidence of thrombosis in those patients was reported (Goodwin et al., 2011). Likewise in people, while a wider array of haemostatic abnormalities affecting both coagulation and platelet function have been identified, very little is known about how they correlate with risk of thrombosis, or which are most useful for detecting a prothrombotic state in individual patients (Giannotta et al., 2015; Purnak and Yuksel, 2015).

1.4 Urinary thromboxanes

As outlined above, platelet activation is a key factor in thrombus formation, however the main ways of monitoring platelet activation are only available in research settings. As urine is an easy sample to collect from clinical cases, measurement of urinary thromboxanes as a marker of thromboxane production may represent an easily accessible marker of platelet activation, and potentially of thrombotic risk.

1.4.1 Thromboxane physiology and measurement

As mentioned above, the phospholipase A_2 pathway is a key platelet metabolic pathway, which plays a key role in platelet activation through generation of thromboxane A_2. As such, measurement of thromboxane A_2 and its downstream metabolites has been widely investigated as a potential marker of platelet activation and thrombotic risk in human medicine, and to a more limited degree in veterinary medicine. In terms of physiology, Thromboxane A_2 is produced from arachidonic acid via the phospholipase A_2 pathway. An overview of this pathway is shown in Figure 1. The arachidonic acid is released from the platelet cell membrane by phospholipase A_2, which is activated by the increase in intracellular calcium concentrations that results from activation of the myriad of platelet surface receptors by their agonists. The two other key enzymes in the synthesis of thromboxane are cyclooxygenase (COX) and thromboxane synthase. Once produced, thromboxane A_2 is released from the platelets into serum, but is extremely unstable, and rapidly breaks down to form thromboxane B_2.

In turn thromboxane B_2 is then metabolised to two products; 11-dehydrothromboxane B_2 (11-dTXB) and 2-3-dinor-11-dehydrothromboxane B_2. These products are both excreted unchanged in urine, with 11-dTXB being the most abundant.

Studies have shown that urinary 11-dTXB excretion is a good estimate of thromboxane production in both people and in dogs (Yamanaka et al., 1993). In early studies 11-dTXB was measured by gas chromatography and mass spectrometry, but since the 1990's enzyme immunoassays have been more commonly used (Reinke, 1992) . The enzyme-based ELISA assays have been validated for use in both human and canine urine (Lellouche et al., 1990; Yamanaka et al., 1993). Urinary 11-dTXB is usually normalised to urine creatinine concentration to give a urine 11-dTXB-to-creatinine ratio (u11-dTXB:Cr). Urinary 11-dTXB has also been shown to be stable at room temperature for up to 6 days after collection (Pagliaccia et al., 2014). While serum thromboxane B2 can be measured, the levels can be artifactually increased by further platelet activation ex-vivo during venepuncture or in blood collection tubes. As such, u11-dTXB:Cr has the advantage of being a more reliable measure of in vivo thromboxane levels, since ex-vivo generation of thromboxane does not occur in urine.

It is important to note that both COX and thromboxane synthase can be expressed in a number of cell types other than platelets, and that several extra-platelet sources of thromboxane have been identified, including the endothelium and activated inflammatory cells. Under normal physiological conditions, between 70 and 80% of human serum thromboxane is produced by platelets, with monocytes being the main extra-platelet source. The proportion of thromboxane produced by monocytes has been shown to be increased in various inflammatory states, in particular by inflammation associated with atherosclerotic plaques (Catella and Fitzgerald, 1987). Since thromboxane is both produced by platelets, and causes platelet activation via surface thromboxane receptors, it has the potential to be useful as a marker of platelet activation for two reasons; firstly as in the absence of significant levels of production from extra-platelet sources thromboxane represents a measure of the degree of metabolic platelet activity; secondly as one of the direct causes of platelet activation, thromboxane concentration can be taken as a marker of the level of stimulation of platelets, irrespective of the source of thromboxane. As such, urinary thromboxane measurement may have utility in detecting prothrombotic states in which platelet activation is occurring.

1.4.2 Urinary thromboxanes in human medicine

In human medicine, u11-dTXB:Cr is a well-established marker of platelet activation, and has been widely evaluated as a biomarker for risk of future thromboembolic disease. It is increased in multiple conditions in which there is an increased thrombotic risk, including atherosclerosis, acute coronary syndrome, stroke, heart failure, atrial fibrillation, venous thromboembolism, diabetes,

hypercholesterolaemia, essential hypertension, chronic kidney disease, rheumatoid arthritis, systemic lupus erythematosus and IBD. While it is not yet used on a day-to-day clinical basis, urinary thromboxanes have been proposed as a future tool to guide the need for antiplatelet prophylaxis(Neath et al., 2014). A u11-dTXB:Cr level above a certain cut-off has already been found to be prognostic for the risk of repeat thrombotic cardiac events in patients with existing coronary artery disease on treatment with aspirin (Vasudevan et al., 2018).

Aside from being a marker of platelet activity, urinary thromboxanes have also been used to evaluate the impact of aspirin therapy on COX inhibition. Aspirin irreversibly acetylates COX and so inhibits its function in a variety of cell types. In platelets, this leads to reduced function and so reduced thromboxane production for the duration of the platelet's lifespan, and so an anti-thrombotic effect clinically. The antithrombotic effect of aspirin has been found to be highly variable in people, with some patients being identified as 'poor responders' to therapy. Since urinary thromboxane production is dependent on COX activity, u11-dTXB:Cr has also been used as a tool to evaluate patients' response to aspirin therapy (Patrono, 2003).

1.4.3 Urinary thromboxanes in veterinary medicine

In veterinary medicine, there have been more limited studies investigating the utility of u11-dTXB:Cr in a clinical setting. The first study evaluating it in dogs looked at its potential utility as a marker of reperfusion of ischaemic gut in dogs with gastric dilation volvulus (GDV). They found both higher u11-dTXB:Cr levels in dogs with GDV overall compared to healthy controls, and higher postoperative levels in those dogs that had complications than those that did not. The preoperative levels were not, however, predictive of outcome (Baltzer et al., 2006). u11-dTXB:Cr has also been used in several studies in healthy dogs as a tool for assessing the effects of aspirin at inhibiting COX. These studies have found that aspirin does decrease u11-dTXB:Cr, as would be expected with successful COX inhibition, but that the effect is very variable, in some studies requiring dosages of 10mg/kg/day (Hoh et al., 2011). Other than the study in dogs with GDV, however, to the authors knowledge there have been no studies looking at u11-dTXB:Cr in naturally diseased dogs. As such nothing is known as to whether u11-dTXB:Cr may be increased in similar situations in dogs to those it is increased in in people, nor whether it may be a marker of platelet activation and thrombotic risk in dogs with prothrombotic conditions.

1.5 Aim of this study

As has been outlined in this introduction, IMHA, PLN and PLE are three commonly seen canine diseases with frequent thromboembolic complications. Research so far has identified a number of pathophysiological abnormalities that underlie this prothrombotic tendency. To date, there is unequivocal evidence that increased platelet activation plays a role in patients with IMHA, but limited work evaluating a role in PLE and PLN. Overall it is likely that a combination of abnormalities to platelet function, secondary haemostatic proteins, and endothelial function contribute to the overall degree of prothrombotic state in an individual patient. Equally it is likely the level of thrombotic risk varies between patients, and within the same patient over the time course of a particular disease. At present we have very limited means of testing patients for a prothrombotic state, and it is unclear whether the results of the tests we do have can be translated to a clinically relevant risk of thrombus formation. Identifying new biomarkers of thrombotic risk that can be easily measured in clinical patients would allow further longitudinal research to correlate test results with the incidence of thrombosis in such patients. Should a reliable biomarker of thrombotic risk be identified, it would enable us both to better stratify patients as to their individual risk, and to help judge whether any antithrombotic therapy prescribed is effective. Urinary thromboxanes have been identified as measures of platelet activation and are easily measured via ELISA. More importantly in human medicine they have been found to correlate with the clinical risk of thrombosis in various situations. They have not, however, to date been assessed for utility in this role in canine patients.

Given platelet activation has been well documented in patients with IMHA, this study's first hypothesis is that urinary thromboxanes as measured by u11-dTXB:Cr are increased at baseline in dogs with IMHA compared to normal controls. The second hypothesis is that since thromboembolic disease is a major cause of mortality in dogs with IMHA, u11-dTXB:Cr will be correlated with survival outcome, or other known markers of disease severity in these patients. A third hypothesis is that since urinary thromboxanes are markers of platelet activation, and platelet activation appears to be important in the pathogenesis of thrombosis, u11-dTXB:Cr will also be higher in dogs with thromboembolic complications of IMHA compared to those without. The final hypothesis for the IMHA patients is that the thrombotic risk varies over the course of their disease, and so a final aim is to describe the change in u11-dTXB:Cr over the first 6 weeks of treatment by serial measurement at predetermined time intervals.

For both PLN and PLE, there has been very limited investigation into the role of platelet activation in thrombosis. However there is evidence from the human field that platelet activation occurs in patients with similar diseases, and may be an important contributor to the prothrombotic state in some of

these patients. The final two hypotheses for this study are that platelet activation is similarly important in veterinary PLN and PLE patients, and so urinary thromboxanes, as measured by u11-dTXB:Cr are increased at baseline in dogs with PLN, and in dogs with PLE.

2 Materials & Methods

2.1 Study design

The main part of this study was a prospective case-control design. Based on a power calculation using u11-dTXB:Cr levels reported in a previous study of pre-neuter control dogs and dogs with gastric dilation-volvulus , an α of 0.05 and a β of 0.9, we aimed to recruit a minimum of 6 normal dogs and 6 'diseased' dogs for each of IMHA, PLN and PLE groups for the basic hypotheses. For the second and third hypotheses, as the range of urinary thromboxane levels likely to be seen in the IMHA dogs was unknown, we aimed to recruit 20 dogs based on reported mortality rate for IMHA of approximately 50%, to give 10 IMHA dogs in each survival group. We estimated based on recent experience that the SAH sees between 10 and 15 canine IMHA cases per year, so planned to recruit cases over a 2 year period.

2.2 Ethical considerations

This study was approved by the University of Glasgow School of Veterinary Medicine Research ethics committee (application 49a16). For the first part the study involved free-catch urine sample collection from all groups of dogs, unless a cystocentesis sample had already been collected as part of the routine work-up, in which cases any excess sample was used. For the second and third parts of the study, most clincopathological data were obtained as part of the standard clinical evaluation of cases. Where possible, residual EDTA blood or serum samples were stored at the time the case was seen, so if required for the study extra biochemical tests or C-reactive protein (CRP) could be run at a later date.

2.3 Study populations

2.3.1 Control group

2.3.1.1 Selection criteria

Control dogs were healthy animals owned by staff and students at the Small Animal Hospital. They were judged to be healthy based on owner history, clinical examination, and routine urinalysis. Dogs were excluded if there were abnormalities on clinical examination, if they had been vaccinated in the preceding 2 weeks, or if they were on long-term medications other than routine endo- and ecto-parasiticides. Any dogs receiving corticosteroids or NSAIDS in the preceding two weeks were likewise excluded.

2.3.1.2 Data recording and sample collection

Signalment data and health information were collected by means of an owner questionnaire. All normal dog urine samples were collected by the owners by free catch.

2.3.2 IMHA group

2.3.2.1 Selection criteria

Dogs with primary immune-mediated haemolytic anaemia were prospectively recruited. Most cases of IMHA present to the referral hospital as emergency cases with anaemia, so were expected to be easily identifiable for possible study inclusion. Dogs were excluded if they had been on immunosuppressive therapy from their referring veterinarian for over 48 hours at presentation. Diagnostic criteria for IMHA were anaemia with HCT <37%, plus the presence of two of three criteria: spherocytosis, positive Coombs test, or positive in-saline agglutination. Dogs were classified as having primary IMHA following exclusion of possible triggers for secondary IMHA; this involved a medical work up as felt indicated by the primary case clinician, and included abdominal and thoracic imaging, further biochemical analysis, urinalysis and infectious disease testing based on travel history. Although the recent ACVIM guidelines for diagnosis of primary IMHA were not published at the inception of this study, these criteria are in line with those recommendations (Garden et al., 2019).

2.3.2.2 Data recording and sample collection

Haematology, serum biochemistry, CRP and fibrinogen were measured at admission as part of the standard case investigation. Depending on the timing of case presentation, the haematology was either run directly at the reference laboratory (ADVIA 120, Siemens, Frimley, UK), or performed on the in-house analyser (Idexx Catalyst). Similarly, biochemistry was either run at the reference laboratory (Dimension XPAND, Siemens, Frimley, UK), or on the in-house analyser (Idexx Procyte). For platelet count, where an automated machine count was not provided due to platelet clumping, clinical pathologists reported a comment on approximate count from blood smear examination. These comments were translated into an estimated platelet count for statistical analysis as per Table 3. The presence or absence of spherocytosis as judged by a clinical pathologist, and results of slide agglutination and direct Coombs tests were also recorded. Urine was collected by free catch within 24h of admission unless cystocentesis was required for the clinical work-up. Each urine sample used for urinary thromboxane measurement was also analysed routinely, and the results of dipstick and sediment exam recorded. For disease severity scoring, CHAOS score, total bilirubin (T.bil), creatinine, urea, ALT, CRP, fibrinogen, HCT, PLT, and ASA score at admission were recorded when available for all cases. CHAOS scores were calculated as previously described (Goggs et al., 2015)(Whelan et al.,

2006)(Table 2). ASA scores were recorded at admission by either the primary case clinician, or an ECC clinician assisting with case management. A coagulation profile was requested for all cases, to include PT, aPTT, FDP, D-dimers and fibrinogen levels. Treatment protocols were recorded for all cases, including dosages of immunosuppressive and anti-thrombotic medications. All therapeutic decisions regarding patient care were made by the primary case clinician as is normal hospital policy; there was no standardisation of therapy as part of this study.

Table 3: Blood smear platelet comment interpretation

Blood smear platelet comment	PLT count range estimate (x10^12/l)	PLT value for statistical analysis (x10^12/l)
adequate	50-200	125
reference	200-500	350
increased	500+	750

Abbreviations: PLT, platelet.

Outcome measures were recorded as survival to hospital discharge, survival to 30 days following admission, and survival to 90 days following admission. Situational details regarding death were recorded for all cases, including whether dogs died naturally or were euthanised. If surviving dogs had returned to the care of their referring veterinarian by 90 days, they were followed up for survival status by telephone. For surviving dogs returning to us for follow-up, we aimed to collect urine samples for repeat measurements of u11-dTXB:Cr at each recheck appointment within the first 6 weeks, to describe the changes in u11-dTXB:Cr over time in these patients. All urine samples from recheck visits were collected by free catch either at the appointment, or within 12 hours of the appointment by the owners at home. Recheck appointment times were not standardised, but were scheduled as deemed necessary by the case clinician. As such sample timings were recorded as day post diagnosis, so they could later be grouped into time intervals for analysis. The planned groupings were; 7 days (+/-2 days), 14 days (+/- 2 days), 28 days (+/- 2 days) and 42 days (+/- 4 days) post-diagnosis. The proportion of these samples that remained above the upper end of the u11-dTXB:Cr range for the normal dogs was described. Records were also assessed retrospectively for any suspicion of thromboembolic disease during hospitalisation, and dogs grouped into those with and without suspicion of thrombosis. Criteria for suspicion of thrombosis included gross thrombi or evidence of infarction seen on imaging or at post mortem, unexplained tachypnoea suspicious for PTE, and neurological signs suspicious for central nervous system (CNS) thrombosis.

2.3.3 PLN group

2.3.3.1 Selection criteria

Dogs with possible PLN were prospectively recruited. However, unlike IMHA cases which usually present with acute anaemia, PLN cases can have a variety of presenting complaints. This can include investigation of pre-identified proteinuria, hypoproteinaemia, abdominal or pleural effusions, subcutaneous oedema, alongside more non-specific complaints such as lethargy, hyporexia, PUPD and vomiting. As such it was expected that some cases would be identified at the point of admission to the hospital, while others may only be identified after discharge, when the results of all investigations became available. For cases included in this study, investigations were performed as deemed necessary by the case clinician to establish a diagnosis of PLN, as is standard hospital policy. Proteinuria was defined as a UPC > 0.5 as per current ACVIM consensus guidelines (Lees et al., 2005). We aimed to include only PLN cases with primary PLN, as some conditions that can underlie secondary PLN such as certain neoplasia and parasitic infections have themselves been associated with hypercoagulability and thrombotic disease (deLaforcade et al., 2019; Williams et al., 2017). Renal histopathology was not a requirement for study inclusion.

2.3.3.2 Data recording and sample collection

Standard signalment, biochemical data as was available, and final diagnosis were recorded for all cases. All urine samples for the study were collected by free catch, unless cystocentesis was required for the clinical work-up. In addition to urine being saved for thromboxane analysis, all cases had a routine urinalysis, urine protein-creatinine ratio (UPC) and bacterial culture as part of their clinical work-up for PLN, the results of which were recorded.

2.3.4 PLE group

2.3.4.1 Selection criteria

Dogs with possible PLE were prospectively recruited. As with PLN, dogs with PLE can have a variety of presenting problems, but most commonly present with chronic diarrhoea. Other potential reasons for case presentation include known hypoalbuminemia, abdominal or pleural effusions or subcutaneous oedema (Craven and Washabau, 2019). Inclusion criteria for a diagnosis of PLE for this study were 1) a history of GI disease (any of weight loss, vomiting, diarrhoea, hyporexia 2) hypoalbuminemia (albumin <29g/l). 3) exclusion of acute GI parasitic or bacterial disease 4) exclusion of hepatic dysfunction. 5) absence of proteinuria. Ideally cases would also have histopathological confirmation of a disease process known to be associated with PLE, although this was not essential. All cases underwent investigation as deemed necessary by the case clinician.

2.3.4.2 Data recording

Standard signalment, biochemical data as was available, and final diagnoses were recorded for all cases. Urine was obtained from all cases prior to any treatment with corticosteroids or other immunosuppressive medications. Again urine samples were collected by free catch, unless cystocentesis was deemed necessary as part of the clinical work-up.

2.4 Urine sample processing and storage

Urine samples from all dogs were frozen at -20°C within 24 hours of collection in 1ml aliquots. All urine was then transferred to storage at -80°C until batch analysis at a later date. Previous studies have shown storage at room temperature for up to 6 days leads to no significant change in u11-dTXB concentrations, and it is stable long-term at -20 to -80°C (Lellouche et al., 1990; Neath et al., 2014).

2.5 UTXB ELISA protocol

Urinary 11-dTXB levels were measured with a commercial monoclonal ELISA kit[2] previously validated for use in canine urine (Baltzer et al., 2006). The assay is an acetylcholinesterase (AChE) competitive ELISA. Briefly, this works on the basis of competition between 11-dTXB in the sample and a tracer of 11-dTXB bound to acetylcholinesterase (11-dTXB_2-AChE) for binding to a monoclonal antibody that is fixed to the ELISA plate. In each well the amount of tracer that can bind to the monoclonal antibody is inversely proportional to the amount of 11-dTXB present in the sample added. Once the plates have been washed, the amount of bound tracer can be determined by addition of a substrate for AChE (Ellmans Reagent). The metabolism of this substrate by the bound AChE causes production of a yellow coloured substance. The intensity of the yellow colour produced can be measured using a spectrophotometer, with samples with greater intensity containing proportionally more tracer and so less 11-dTXB from the sample. Conversely, samples with high levels of 11-dTXB outcompete the tracer to a greater extent, and so less yellow colour develops. The ELISAs were performed as per the manufacturer instructions as follows: Urine samples were defrosted to room temperature and centrifuged at 3000rpm for 5 minutes to remove precipitated proteins prior to dilution. The first samples were all run at 3 dilutions (1:2, 1:4, 1:8), however once a realistic range of the 11-dTXB level had been established for the IMHA dogs, subsequent samples for IMHA cases were run at 2 dilutions (1:5 and 1:10). Any samples with results still falling outside the linear region of the standard curve at

[2] Cayman chemical 11-dehydo Thromboxane B2 ELISA Kit0 Monoclonal. Item number 519510. Cayman Chemical, Ann Arbor, MI, USA

these dilutions were re-run on a subsequent plate at adjusted dilutions (1:75 and 1:150). All sample dilutions for all dogs were run in duplicate. Samples were diluted with assay buffer and incubated at 4°C overnight. The ELISA plates were set up, incubated at room temperature for 2 hours, and then developed as per manufacturer instructions. The plates were read using an automated plate reader[3] at 405nm wavelength after 40 to 60 minutes when the maximum tracer binding (Bo) well absorbance was in the range of 0.3-1.5 AU. Standard curves were calculated, and results processed using commercially available software[4]. Results from the ELISA were reported in pg/ml. The assay has a measurable range from 15.6-2,000 pg/ml. Results were corrected for dilution by multiplication by the dilution factor, and converted to ng/ml. Where multiple dilutions gave valid results within the working range of the assay, these were averaged to give a final 11-dTXB concentration (ng/ml) for each urine sample. Any results outside the working range of the assay were discarded. The mean urine 11-dTXB concentration (ng/ml) was then normalised to the urine creatinine to give a urinary 11-dTXB to creatinine ratio (u11-dTXB:Cr). Urine creatinine was measured at a reference laboratory by the Jaffe reaction method and reported in umol/l (Jaffe, 1886), but was converted to mg/ml, so as to report the final u11-dTXB:Cr as ng/mg crea as in previous studies.

2.6 Statistical analysis

Data recording and statistical analysis was performed using commerically available spreadsheet management[5] and statistics package[6] software. Urinary 11-dTXB:Cr results for all disease groups, and IMHA subgroups were assessed for normality via visual inspection of histograms and normal Q-Q plots, plus Kolmogorov-Smirnov tests. Data that were normally distributed were compared between groups using independent-sample T tests. Non-normally distributed data were compared using Mann-Whitney tests, and medians reported.

For the three basic hypotheses, u11-dTXB:Cr for the dogs with IMHA, PLE and PLN was compared with the normal controls using either independent-sample T tests, or Mann-Whitney tests as appropriate based on normality testing.

To investigate correlations between u11-dTXB:Cr and survival for IMHA, u11-dTXB:Cr at initial admission was also compared using Mann-Whitney tests between IMHA survival outcome groups. The survival outcome groups analysed were; surviors and non-survivors at hospital discharge, survivors

[3] LT-4500 Automatic microplate absorbance reader. Labtech, Heathfield, UK
[4] Assayzap. Biosoft, Cambridge, UK
[5] Excel, Microsoft Office 2016, Microsoft
[6] IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY

and non-survivors at 30 days post presentation, and survivors and non-survivors at 90 days post presentation. To investigate correlations between u11-dTXB:Cr and disease severity for IMHA, initial values for parameters previously associated with either disease severity or survival (CHAOS score, T.bil, creatinine, urea, ALT, CRP, fibrinogen, HCT, PLT, WBC, ASA score at admission) were firstly compared between just the 30 day survivors and non-survivors, as descriptive analysis of the IMHA population. For continuous variables Mann-Whitney tests were used for analysis, for the categorical scores (CHOAS and ASA) Chi-squared tests were used to compare u11-dTXB:Cr between dogs with scores ≥3 and <3. These categories were chosen based on previous studies (Goggs et al., 2015). Where a stastically significant difference between the groups was identified using Mann-Whitney analysis, univariable binary logistic regression was used to further quantify the magnitude of effect. Correlations between u11-dTXB:Cr and the continous markers of disease severity for the IMHA group as a whole were also assessed by means of visual assesment of scatter plots, and linear regression analysis. Where the relationship between variables was non-linear based on visual assessment of scatter plots, or residuals failed the assumptions of normal distriibution and even variance based on histogram and pp-plot analysis, the variables were log-transformed to achieve a linear relationship.

To evaluate u11-dTXB:Cr in dogs with thromboembolic complications of IMHA compared to those without, u11-dTXB:Cr at initial admission was compared between groups using Mann-Whitney tests.

The final aim was to describe the change in u11-dTXB:Cr over the first 6 weeks of treatment. Due to anticipated low case numbers that would preclude statistical analysis, this part of the study was planned to be purely descriptive. Trends in Individual patient u11-dTXB:Cr were graphed by timepoint. Median and ranges for u11-dTXB:Cr at each grouped timepoint were calculated. If sufficient numbers were available, comparison of average u11-dTXB:Cr between each grouped timepoint and the normal controls would be performed, or the proportion of dogs with results above the highest control dog u11-dTXB:Cr value described.

3 Results

3.1 Population characteristics

3.1.1 Healthy dog population characteristics

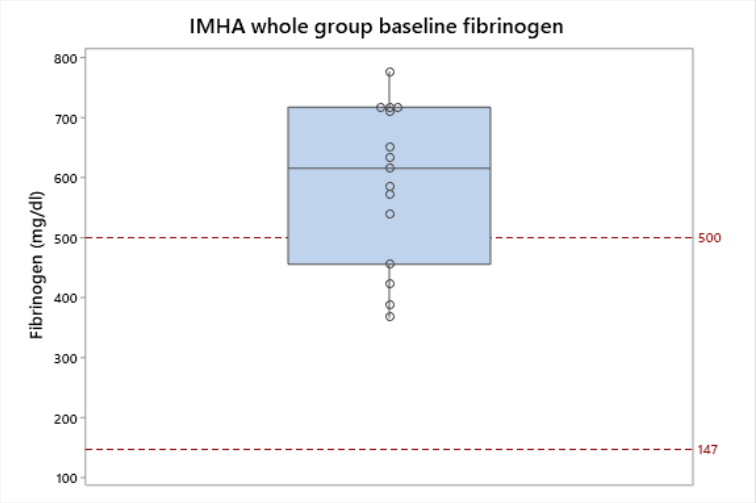
Seventeen healthy dogs were volunteered, of whom one was excluded for otitis externa and osteoarthritic joint disease. Thus 16 'normals' were included in the study. These were a range of breeds, of which three were Springer Spaniels. Ages ranged from 9 months to 11 years with a mean of 5.2 years.

3.1.2 IMHA dog population characteristics

Twenty-four dogs with suspected primary IMHA were recruited to the study. Two dogs were excluded, one because he was subsequently diagnosed with phosphofructokinase deficiency, and one because the IMHA was secondary to neoplasia. Two additional dogs did not have urine collected within 24h of admission, so were likewise excluded. Thus twenty dogs with primary IMHA were included in this study. There were a range of breeds, including 3 Cocker Spaniels, 2 Border Collies, 2 Springer Spaniels, 2 crossbreeds, and one each of Labrador, Whippet, Bichon Frise, Cairn Terrier, Irish Setter, French Bulldog, Cockerpoo, Miniature Schnauzer, Jack Russel Terrier, Labradoodle and Pomeranian. The dogs ranged in age from 2 to 12 years, with a mean of 7.5 years. The mean age of the IMHA dogs (7.5 years, SE Mean 0.65) was not statistically significantly higher than the normal dogs (mean 5.5 years, SE Mean 0.77) ($p=0.06$).

Selected basic clinicopathological data is shown in Table 4, full data can be found in Appendix 1. Of note, full clotting profiles including FDP and D-dimers were only performed in 4 of twenty dogs, so further analysis of these data was not performed. Twelve of twenty dogs had CRP measurements available. Notably 11 of the 15 dogs who had fibrinogen measured had a concentration above the laboratory reference range (147-500 mg/dl) (Figure 3).

Figure 3: Box and Whisker plot of baseline fibrinogen concentrations for 15 dogs with primary IMHA. Red dotted lines represent laboratory upper and lower reference intervals. Box represents 75th percentile, median and 25th percentile. Circles represent individual data values.



All dogs were treated with corticosteroids, with 17 starting therapy with intravenous dexamethasone, at a mean dose of 0.32mg/kg/day (range 0.07-0.6mg/kg/day) and three starting therapy with oral prednisolone, at a mean dose of 1.65mg/kg/day (range 1.01-2.0mg/kg/day). All but one dog received a second line immunosuppressive drug prior to discharge from hospital; twelve dogs were treated with mycophenolate at a mean dose of 14mg/kg/day (range 8.1-24.3mg/kg/day), six with cyclosporine at a mean dose of 8mg/kg/day (range 4.0-16.3mg/kg/day), two with azathioprine (dosages 2-2.1mg/kg/day), and one dog received intravenous human immunoglobulin (0.5g/kg). Overall one dog was treated with a single immunosuppressive agent, seventeen dogs treated with two agents, one dog with three agents, and one dog with four agents. All dogs received clopidogrel for thromboprophylaxis, at a mean dose of 2.25mg/kg/day (range 0.97-4.7mg/kg/day). Sixteen dogs received one or more blood transfusions; all were given packed red cells. Exact drug combinations and dosages for all individual patients are shown in Appendix 1 - Table 4.

Table 4: Selected biochemical parameters for all IMHA patients at baseline, IMHA patients surviving to 30 days post diagnosis, and IMHA patients not surviving to 30 days post diagnosis

Parameter	All IMHA dogs			IMHA 30 day survivors			IMHA 30 day non-survivors			P-value
	n	median	range	n	median	range	n	median	range	
Age (years)	20	7.5	2-12	11	9	2-12	9	6	4-10	0.34
CHAOS score points (maximum 7)	20	3.6	1-7	11	3	1-7	9	4	2-6	0.24
T.bil (umol/l)	20	137.4	0-667	11	27	4-143	9	294	0-667	0.07
CRP (mg/l)	12	189	41-348	6	137	40-187	6	252	121-348	0.03
HCT (%)	20	16	10-26	11	17	12-26	9	13	10-20	0.03
PLT (x10^12/l)	19	324	101-846	11	333	101-846	8	314	125-499	0.83
Fibrinogen (mg/dl)	15	592	368-777	8	625	368-718	7	572	388-777	1
Creatinine (umol/l)	20	76	35-253	11	59	37-94	9	69	35-253	0.18
Urea (mmol/l)	19	17	5-27	10	8.3	5-112	9	15.4	7-27	0.02
ALT (U/l)	16	325	25-2704	9	36	25-237	7	171	32-2704	0.04
ASA score	20	3.5	2-5	11	3	2-4	9	4	3-5	0.002

Abbreviations: IMHA, Immune mediated haemolytic anaemia; n, number; T.bil, Total bilirubin; CRP, C-reactive protein; HCT, Haematocrit; PLT, Platelet count
Notes: P values are for Mann-whitney comparisons between 30-day survivors and non-survivors.

3.1.3 PLN dog population characteristics

Eight dogs with PLN were recruited to the study between April 2017 and February 2019. These included three Yorkshire Terriers, and one each of Lhasa Apso, Beagle, Dogue de Bordeaux, Labrador and Whippet. The dogs ranged in age from 9 months to 10 years, with a mean of 6.6 years. The mean age of the PLN dogs (6.6 years, SE Mean 1.12) was not statistically significantly different from that of the normal dogs (mean 5.5 years, SE Mean 0.78) (p=0.44).

The dogs presented for a variety of reasons including hypoalbuminaemia (n=6), hypoproteinaemia (n=3), ascites, weight loss, azotaemia, acute blindness, CNS signs (n=2), aortic thromboembolism, and incidentally identified proteinuria. All dogs were proteinuric, with UPC ranging from 1.54 to 31.6, median UPC 10.5. For the 6 hypalbuminaemia dogs, the median serum albumin was 17.5 g/l (laboratory reference range 29-36 g/l), with a range of 14-25 g/l. The two normoalbuminaemic dogs had serum albumin levels of 30 g/l and 33 g/l respectively. Five of the 8 dogs were azotaemic at day 0. The median creatinine for the group was 149umol/l with a range of 60-339umol/l. The median urea for the group was 14.9mmol/l (range 2.5-45.7mmol/l) (laboratory reference range 2.5-8.5 mmol/l). Basic clinicopathological data for the dogs are shown in Table 5. Two of the dogs had suspected or confirmed thrombi at presentation; one had presented for vestibular signs (PLN 04); one had an aortic thrombosis (PLN 08). One additional dog (PLN 02) developed seizures and was euthanized 4 days after presentation; differentials for the seizures included CNS haemorrhage due to hypertension and thromboembolic disease. Post mortem exam was not performed.

Table 5: Basic clinicopathological data for the 8 dogs with Protein losing nephropathy

Study number	Age (years)	Breed	Sex	Weight (kg)	Clinical abnormalities	UPC	Albumin (g/l)	Total Protein (g/l)	Creatinine (umol/l)	Thrombus status	u11-dTXB:Cr (ng/mg crea)
PLN 01	5	Lhasa	Fn	9.9	ascites, proteinuria, hypoalbuminaemia	9.78	15	44	176	not suspected	0.640
PLN 02	4	Beagle	Mn	20.4	acute azotaemia, proteinuria, hypoproteinaemia	31.6	16	44	248	suspected	1.981
PLN 03	10	Yorkshire terrier	Fn	2.3	weight loss, proteinuria, hypoalbuminaemia	7.29	19	54	60	not suspected	0.953
PLN 04	10	Yorkshire terrier	Mn	10.7	vestibular signs, proteinuria	11.29	33	67	122	suspected	1.157
PLN 05	0.75	Dogue de Bordeaux	Me	53	skin lesions, proteinuria	1.54	30	76	114	not suspected	0.567
PLN 06	7	Yorkshire terrier	Fe	3.8	blindness, hypertension, proteinuria	20.5	19	59	339	not suspected	1.548
PLN 07	8	Labrador	Me	28.6	skin disease, proteinuria, hypoalbuminaemia	3.9	25	72	60	not suspected	1.854
PLN 08	8	Whippet	Fn	16.4	hindlimb weakness, proteinuria	11.23	14	39	194	aortic thrombus	0.358

Abbreviations: PLN, Protein-losing nephropathy; UPC, urine protein-creatinine ratio; Fn, female neutered; Mn, male neutered; Me, male entire; Fe female entire

3.1.4 PLE dog population characteristics

Seven dogs with PLE were recruited to the study between August 2017 and November 2018. This included two Border Collies, and one each of a Staffordshire Bull Terrier, Soft Coated Wheaten Terrier, Springer Spaniel, German Short Haired Pointer, and Cocker spaniel. All dogs presented for a history of chronic diarrhoea, most already had hypoalbuminaemia identified by their referring vets. The dogs ranged in age from 4 years to 11 years, with a mean of 8.6 years. The mean age of the PLE dogs (8.6 years, SE Mean 0.90) was statistically significantly higher than the normal dogs (mean 5.5 years, SE Mean 0.78) (p=0.02).

All dogs were hypoalbuminaemic; the median serum albumin was 18g/l (laboratory reference range 29-36g/l) with a range of 14-22 g/l. Folate and cobalamin (B12) levels were measured at presentation in 6 of the 7 dogs. Of those, folate was normal in 3 dogs, and increased in 3. Cobalamin was below the lower end of the laboratory reference range (200ng/l) in one dog, with a median concentration for the group of 158ng/l, range 168 – 1807 ng/l. Basic clinicopathological data for the group is shown in Table 6. Only one dog had a suspicion of thromboembolic disease; she had been having unexplained neurological episodes over the preceding month and had evidence of old renal infarcts on abdominal ultrasound.

Table 6: Basic clinicopathologic data in the 7 dogs with Protein losing enteropathy

Study number	Age (years)	Breed	Sex	Weight (kg)	Clinical abnormalities	Albumin (g/l)	Total protein (g/l)	Folate (ng/ml)	B12 (ng/l)	Thrombus status	u11-dTXB:Cr (ng/mg crea)
PLE 01	8	Staffordshire bull terrier	Me	19.1	Chronic diarrhoea, hypoalbuminaemia	18	34	3.3	168	not suspected	0.621
PLE 02	9	Soft coated wheaten terrier	Mn	14.9	Chronic diarrhoea, hypoalbuminaemia	14	29	3.9	372	not suspected	0.955
PLE 03	8	Springer spaniel	Fn	18.3	Chronic diarrhoea, hypoalbuminaemia	22	53	15	986	not suspected	1.650
PLE 04	4	German short haired pointer	Fn	26	Chronic diarrhoea, hypoalbuminaemia	17	35	>20	1807	not suspected	0.771
PLE 05	9	Cocker spaniel	Fn	9.8	Chronic diarrhoea, hypoalbuminaemia	20	44	>20	485	not suspected	2.189
PLE 06	11	Border collie	Fn	17.8	Chronic diarrhoea, hypoalbuminaemia	14	30			not suspected	1.376
PLE 07	11	Border collie	Fn	15.5	Chronic diarrhoea, hypoalbuminaemia	18	34	3	552	suspected	0.478

Abbreviations: PLE, Protein losing enteropathy; Fn, female neutered; Mn, male neutered; Me, male entire; Fe female entire

3.2 Urine thromboxane results

3.2.1 ELISA performance data

Nine ELISA plates were used to obtain the u11-dTXB results within this study. Intra-plate assay performance data for the nine individual plates is shown in Table 7. Inter-plate performance was not assessed as part of this study, but has ranged from 12- 20% in previous published studies measuring u11-dTXB (Baltzer et al., 2006).

Table 7: Intra-plate performance data for the nine individual ELISA plates used to measure u11-dTXB

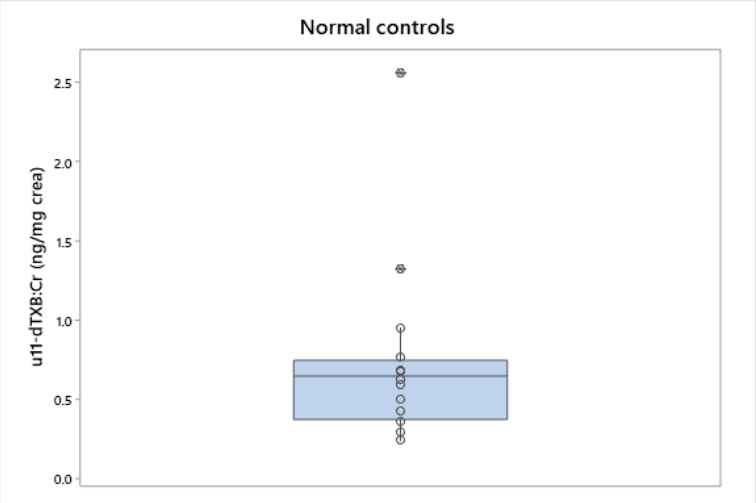
Plate	mean CV	maximum CV
1	5.6	16.6
2	4.1	15.9
3	7.7	23
4	3.5	11.3
5	6.6	17.7
6	3.8	10.2
7	7.6	23.4
8	7.6	10.1
9	6.7	12.9

Abbreviations: CV, coefficient of variation

3.2.2 Healthy controls

The 16 normal dogs had u11-dTXB:Cr results ranging from 0.24 to 2.57 ng/mg crea. These values were not normally distributed (Figure 4). One result was an extreme outlier, so was removed from further analysis (u11-dTXB:Cr 2.57ng/mg crea). The median u11-dTXB:Cr for the remaining 15 normal dogs was 0.62 ng/mg crea (SD 0.28, range 0.24-1.32).

Figure 4: Box and whisker plot of u11-dTXB:Cr for 16 healthy control dogs. Box represents 75th percentile, median and 25th percentile. Circles represent individual data points

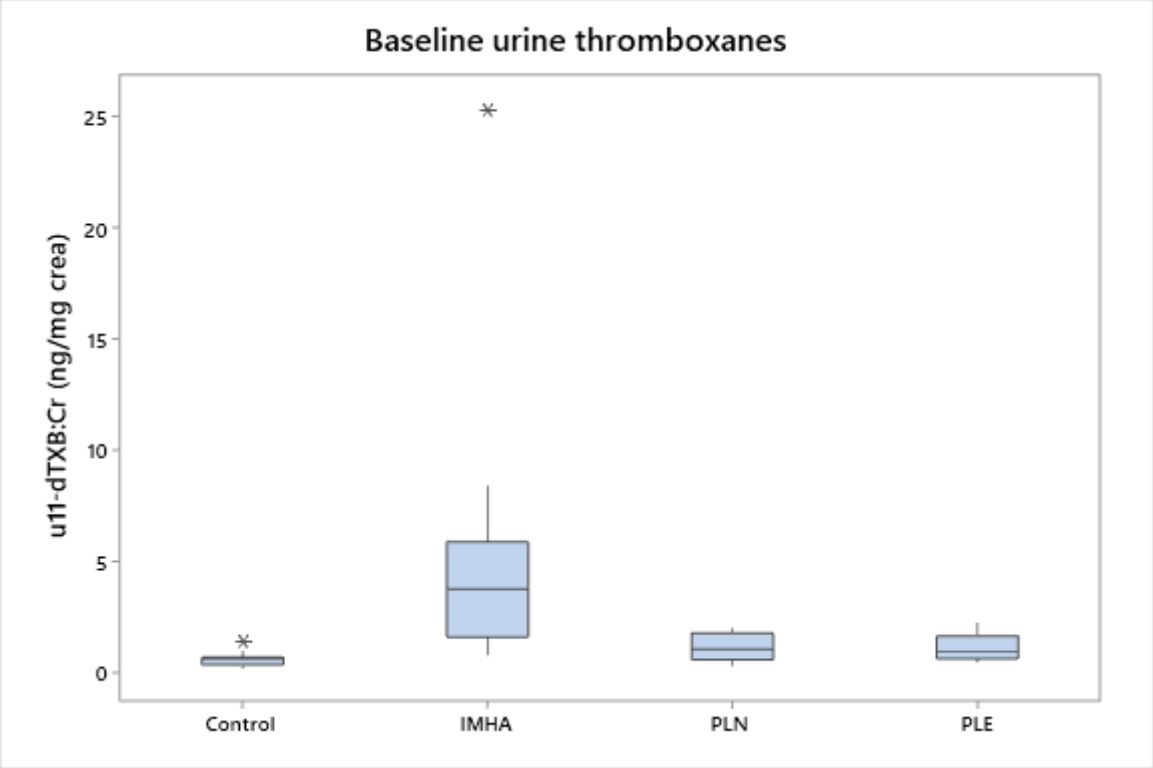


3.2.3 IMHA results

3.2.3.1 IMHA whole group baseline thromboxanes

The 20 IMHA dogs had u11-dTXB:Cr results ranging from 0.83- 25.36 ng/mg crea. These values were not normally distributed. The median baseline u11-dTXB:Cr for the IMHA dogs was 3.76 ng/mg crea (SD 5.34). This was significantly higher than for the normal dogs (median 0.62, SD 0.56, range 0.24-1.32)($p<0.001$) (Figure 5). A post-hoc power calculation using a freely available online tool[7] showed $\beta=0.94$ for this comparison. Thirteen of twenty IMHA dogs had baseline u11-dTXB:Cr higher than the upper end of the range for the normal dogs (1.32 ng/mg crea). Individual results for all IMHA dogs at baseline are shown in Appendix 1.

Figure 5: Box and whisker plots of baseline u11-dTXB:Cr for dogs with IMHA, PLN and PLE compared to healthy controls. Boxes represent 75th percentile, median and 25th percentile. Asterisks represent outliers



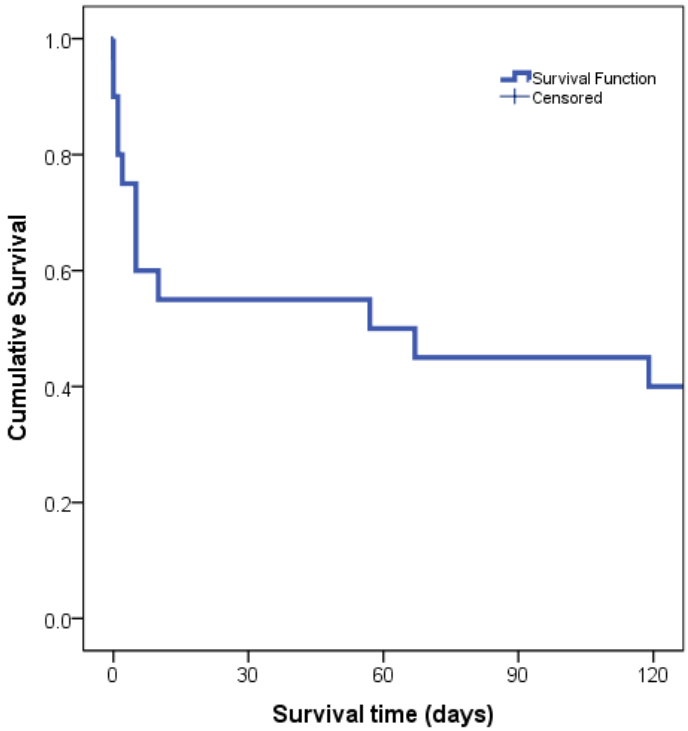
[7] https://clincalc.com/stats/power.aspx

3.2.3.2 IMHA dog baseline subgroup analyses

3.2.3.2.1 Survival outcome subgroups

Of the 20 IMHA dogs, 11 (55%) survived to discharge, giving an in-hospital mortality rate of 45%. Of the non-survivors, 5 died naturally while 4 were euthanised. Of the 4 euthanised, two were euthanised at days 1 and 2 of treatment due to development of neurological signs attributed to thromboembolic disease, while the other two were euthanised after 5 and 10 days of treatment due to ongoing transfusion requirements. Of those surviving to discharge, hospitalisation time was a median of 8 days (range 3-14 days). Two dogs relapsed within 90 days of discharge, one at day 57 post diagnosis and one at day 67 post-diagnosis, both of whom died. This gave a 30-day survival rate the same as the survival to discharge rate, of 55%, and a 90-day survival rate of 45% (Figure 6).

Figure 6: Kaplan Mayer survival curve for 20 dogs with primary IMHA for the first 120 days post presentation to the hospital



There was no significant difference in u11-dTXB:Cr at presentation between dogs with IMHA surviving to 30 days post diagnosis (median 2.20 ±7.2 , range 0.83-25.36)) and those dying within 30 days of diagnosis (median 4.29 ±1.6, range 1.71-6.28)(p=0.45)(Figure 7). Identical results were found for dogs surviving and not surviving to discharge. There was also no significant difference in u11-dTXB:Cr at presentation between IMHA dogs surviving to 90d (median 1.57 ±3.0, range 0.83-8.37) and those not (median 4.29 ±6.6, range 1.71-25.36)(p=0.22)(Figure 8). Post-hoc power calculations, however, showed these comparisons were underpowered, with β=0.08 for the 30d groups, and β=0.18 for the 90d groups.

Figure 7: Box and whisker plots of u11-dTXB:Cr for surviving and non-surviving dogs at 30 days post diagnosis with primary IMHA. Boxes represent 75th percentile, median, and 25th percentile. Asterisks represent outliers.

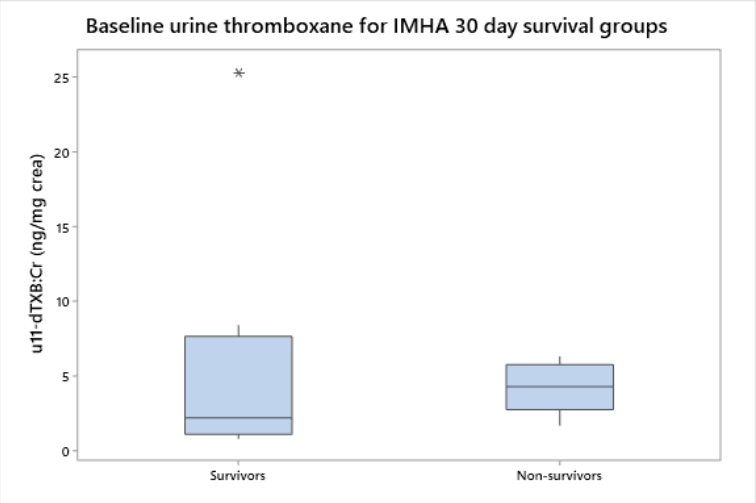
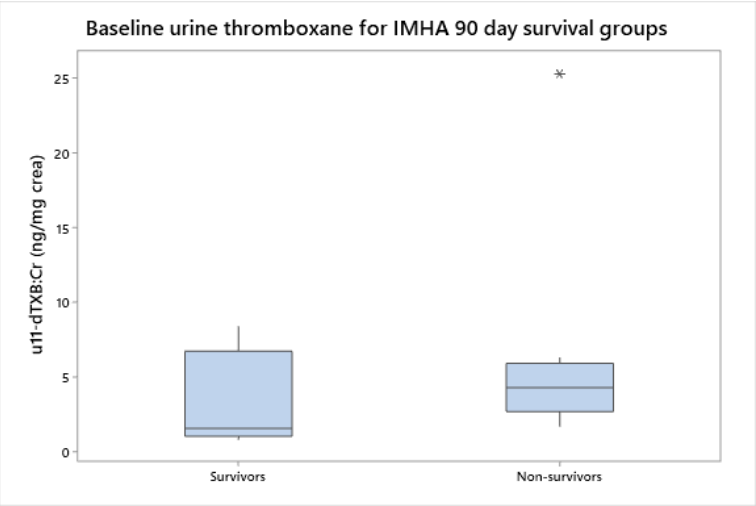


Figure 8: Box and whisker plots of u11-dTXB:Cr for surviving and non-surviving dogs at 90 days post diagnosis with primary IMHA. Boxes represent 75th percentile, median, and 25th percentile. Asterisks represent outliers.



3.2.3.2.2 Disease activity outcomes

Of the variables previously identified as markers of disease severity in IMHA, the median CRP, urea, ALT and ASA score were all significantly higher at presentation in dogs that died within 30d of diagnosis than those surviving to 30d. Additionally HCT was significantly lower in 30d non-survivors than in survivors (Table 4 and Figure 9). To further investigate these relationships, binary logistic regression was performed for survival status at 30d and CRP, urea, ALT, and HCT. Of these, only urea retained statistical significance, with an increase of 1mmol/l urea having an odds ratio of 1.36 for non-survival at 30d (95% CI 1.002-1.860, p=0.049) (Table 7). As only 12 cases had data for CRP, and 16 for ALT, insufficient case numbers were available for multivariable binary logistic regression, so this was not performed. The proportion of dogs with CHAOS score <3 and ≥3 was also compared between 30d survivors and non-survivors using a chi-squared test. Of the 5 dogs with CHAOS score <3, four (80%) were alive at 30d, while of the 15 with CHAOS score ≥3, seven (47%) were alive at 30d. This difference was not statistically significant (p=0.19).

u11-dTXB:Cr was assessed for correlation with the known markers of inflammation and disease severity (CRP, HCT, PLT, CHAOS score, T.bil, Crea, Urea, ALT, WBC, fibrinogen and ASA score) plus age in the 20 dogs with IMHA using linear regression analysis. For all analysis log-transformation of u11-dTXB:Cr was required for the model's assumptions to be valid. No statistically significant correlations between u11-dTXB:Cr and any of the variables were found (Table 8).

Figure 9: Box and Whisker plots of baseline CRP (n = 12) , Urea (n = 19), ALT (n = 16) and HCT (n = 20) for surviving and non-surviving dogs at 30 days post diagnosis with primary IMHA. Boxes represent 75th percentile, median and 25th percentile. Asterisks represent outliers

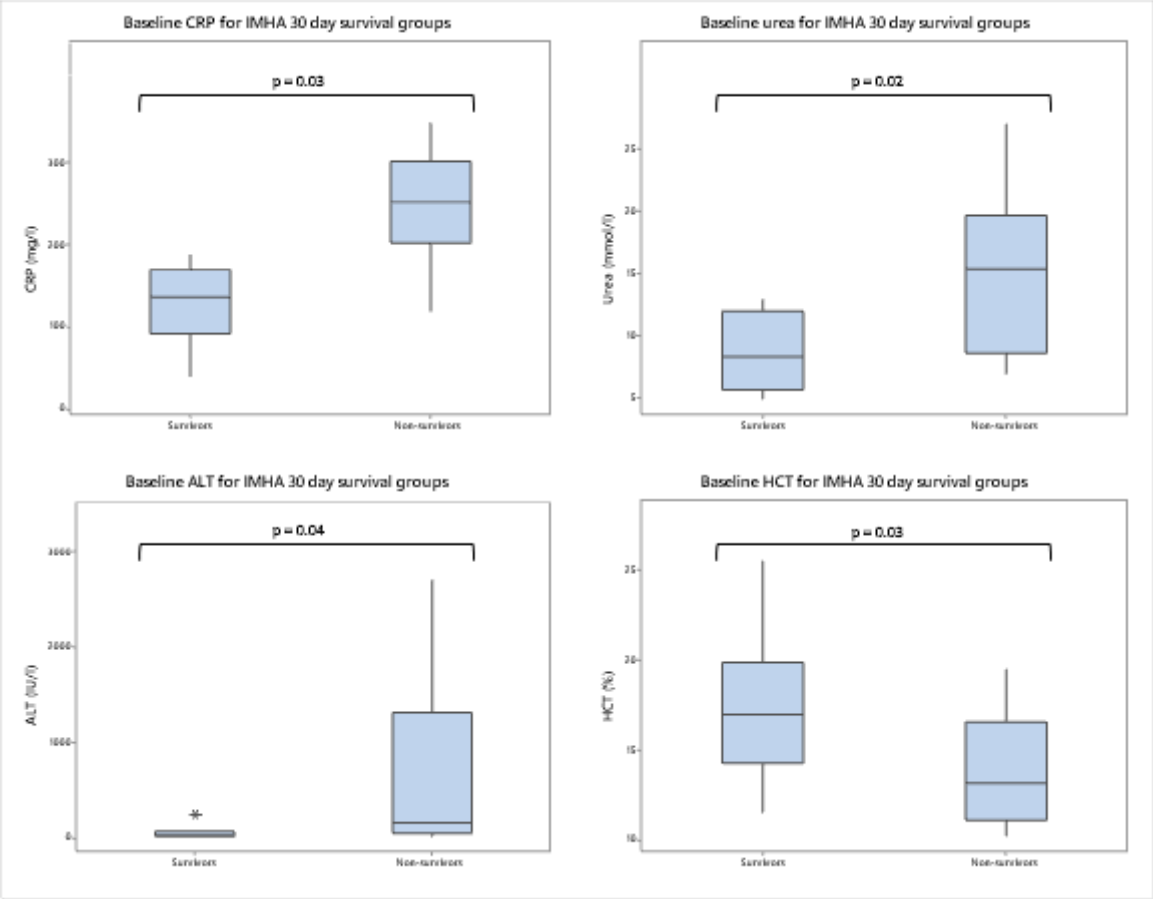


Table 8: Binary logistic regression analysis for selected baseline parameters with survival status at 30 days post diagnosis for dogs with IMHA

Parameter	n (total)	Exp(β)	95% CI for Exp(β)		p-value
			lower	upper	
HCT	20	0.749	0.552	1.016	0.063
CRP	12	0.067	0.998	1.068	0.067
Urea	19	1.365	1.002	1.86	0.049*
ALT	16	1.01	0.994	1.027	0.233

Abbreviations: CI, Confidence interval; CRP, C-reactive protein.
Notes: Exp(β) is the test statistic. * denotes statistical significance

Table 9: Results from linear regression analysis for selected markers of disease severity and urinary thromboxanes in dogs with IMHA

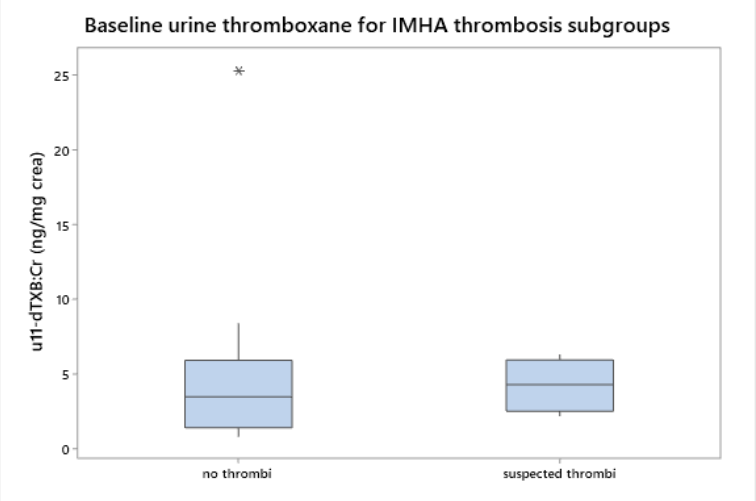
Parameter	n (total)	R^2	β	95% CI for β		p-value
				lower	upper	
Age	20	0.001	-0.01	-0.16	0.14	0.886
CRP	12	0.09	-0.01	-0.009	0.007	0.767
HCT	20	0.011	-0.022	-0.122	0.079	0.658
log(PLT)	19	0	0.021	-0.71	0.752	0.952
log(T.bil)	20	0.012	-0.23	-0.3	0.3	0.867
Creatinine	20	0.001	0	-0.007	0.008	0.898
Urea	19	0.005	0.11	-0.064	0.085	0.076
log(ALT)	16	0.01	0	-0.001	0.001	0.707
WBC	19	0.022	-0.13	-0.55	0.3	0.54
Fibrinogen	15	0.07	-0.002	-0.006	0.002	0.34

Abbreviations: CRP, C-reactive protein; HCT, haematocrit; PLT, platelet count; T.bil, total bilirubin; WBC, total white blood cell count.
Notes: All parameters were assessed against log(u11-dTXB:Cr) to achieve a linear relationship for model validity, normal distribution of residuals and even variance of residuals. * denotes statistical significance

3.2.3.2.3 Thrombosis outcome subgroups

Five of the 20 IMHA dogs had a suspicion of thromboembolic disease during hospitalisation. One dog suffered a sudden respiratory arrest suspected to be a result of PTE, two developed neurological signs including nystagmus and seizures, and two dogs developed a combination of unexplained tachypnoea and neurological signs. Of these dogs, 4 died or were euthanised at the time of the suspected thrombotic event, while one survived to discharge. There was no significant difference between the median u11-dTXB:Cr of these 5 dogs (4.29 ± 1.75, range 2.2-6.3) and the rest of the IMHA group (3.48 ± 6.14, range 0.83-25.4)(p=0.6) (Figure 10). However a post-hoc power calculation gave a β of only 0.07 for these group sizes.

Figure 10: Box and whisker plots of u11-dTXB:Cr for dogs with primary IMHA with and without a suspicion of thromboembolic disease during hospitalisation. Boxes represent 75th percentile, median and 25th percentile. Asterisks represent outliers.



3.2.3.3 Serial measures in surviving IMHA dogs

Of the 11 dogs surviving to discharge, repeat urine samples were obtained at at-least one follow-up timepoint for 9 of them. Exact-day results for individual dogs are shown in Figure 11. Due to more variability than had been anticipated in the actual 'day number' for follow-up visits, the original planned groupings were altered from '7 days (+/-2 days), 14 days (+/- 2 days), 28 days (+/- 2 days) and 42 days (+/- 4 days) post-diagnosis' to three new broader categories, each covering consecutive two-week periods of '2-15 days, 16-29 days and 30 to 42 days'. Eight samples from 7 dogs were available in days 2 to 15 post-diagnosis, eleven samples from 8 dogs in days 16 to 29, and three samples from 2 dogs in days 30 to 42. For the day 2 to 15 interval, no samples were collected sooner than day 6. The median and range for IMHA dogs at each grouped time-point is shown in Figure 12 and Table 9. The median u11-dTXB:Cr for the IMHA dogs was at all time points was statistically higher than that of the normal dogs. The proportion of samples at each time point with u11-dTXB:Cr levels above the upper end of the range of normal dog values (1.32ng/mg crea) is also shown in Table 9 .

Figure 11: Individual dog u11-dTXB:Cr results over time for surviving dogs with primary IMHA

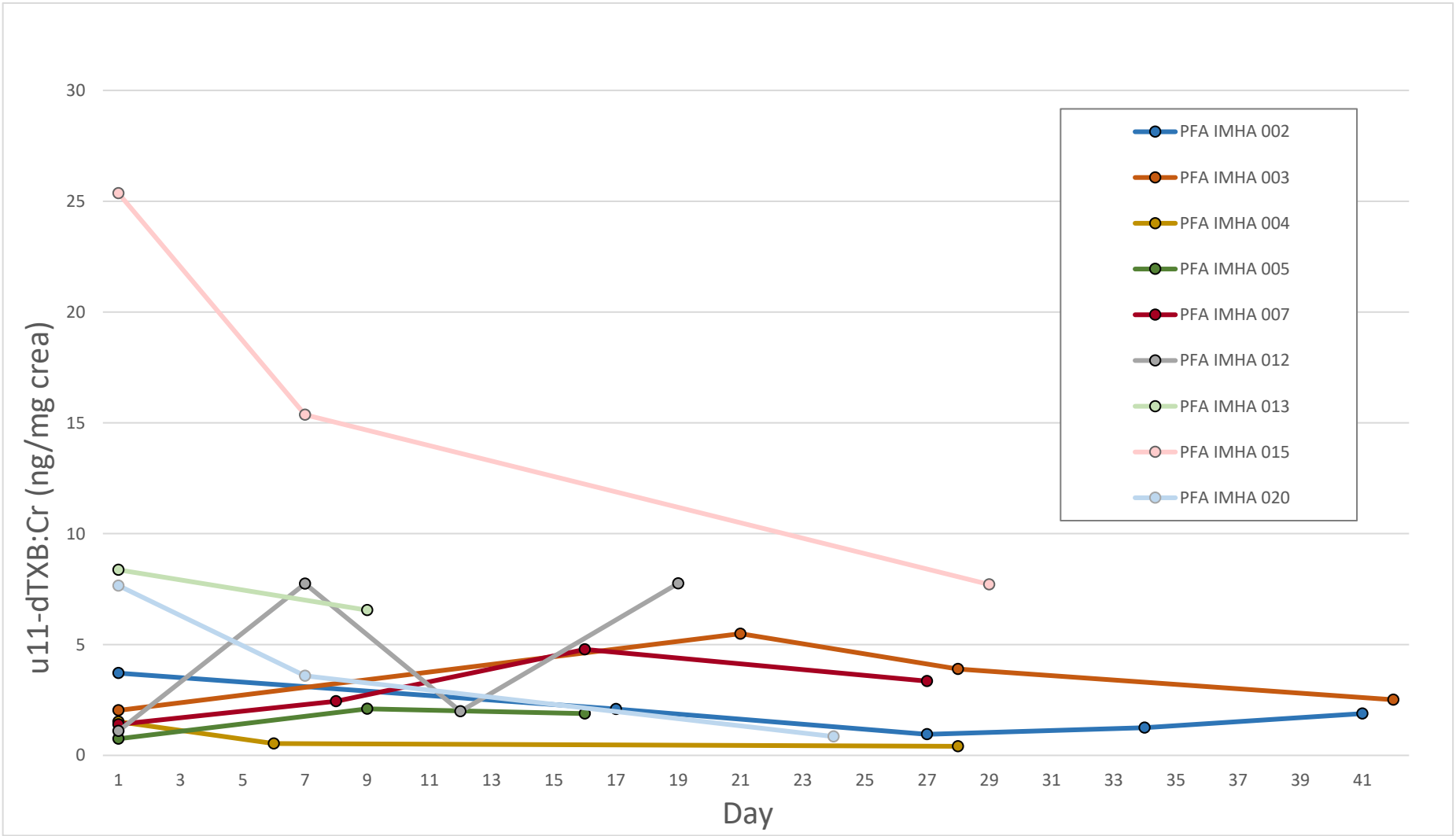


Figure 12: Box and whisker plots of u11-dTXB:Cr for surviving dogs with primary IMHA at serial timepoints post-diagnosis. Boxes represent 75th percentile, median, and 25th percentiles. Asterisks represent outliers. Red dotted line represents upper range value of u11-dTXB:Cr for the control dog group

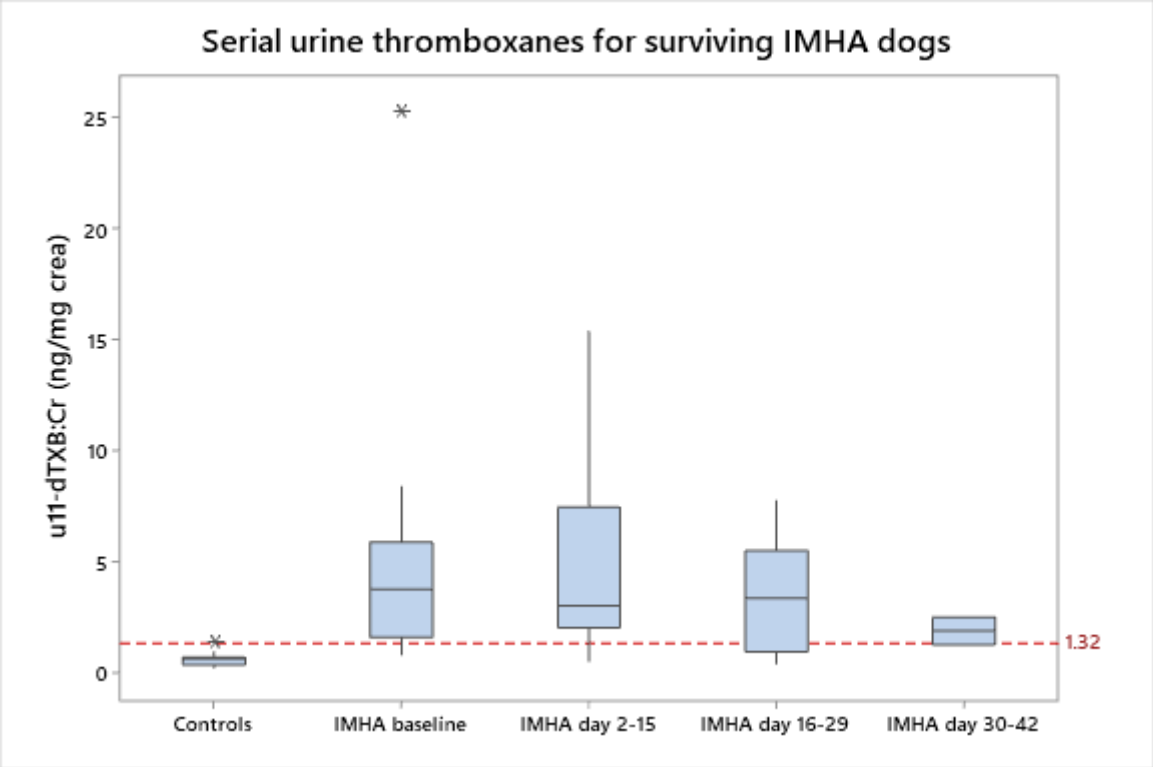


Table 10 : Serial urine thromboxane results for surviving dogs with IMHA at each timepoint

Group	Timepoint	n (results)	n (dogs)	Median u11-dTXB:Cr (ng/mg crea)	Range u11-dTXB:Cr (ng/mg crea)		P-value	Proportion results high
					lower	upper		
controls	day 0	15	15	0.62	0.24	1.32	n/a	n/a
IMHA	day 0	20	20	3.76	0.83	25.4	<0.001*	17/20
	day 2 - 15	8	7	3.02	0.53	15.4	0.001*	7/8
	day 16 - 29	11	8	3.35	0.41	7.76	<0.001*	8/11
	day 30 - 42	3	2	1.88	1.25	2.51	0.011*	2/3

Notes: p-values are for comparison with baseline control group median u11-dTXB:Cr. * denotes statistical significance. High results are as compared to the highest control dog u11-dTXB:Cr value (1.32 ng/mg crea)

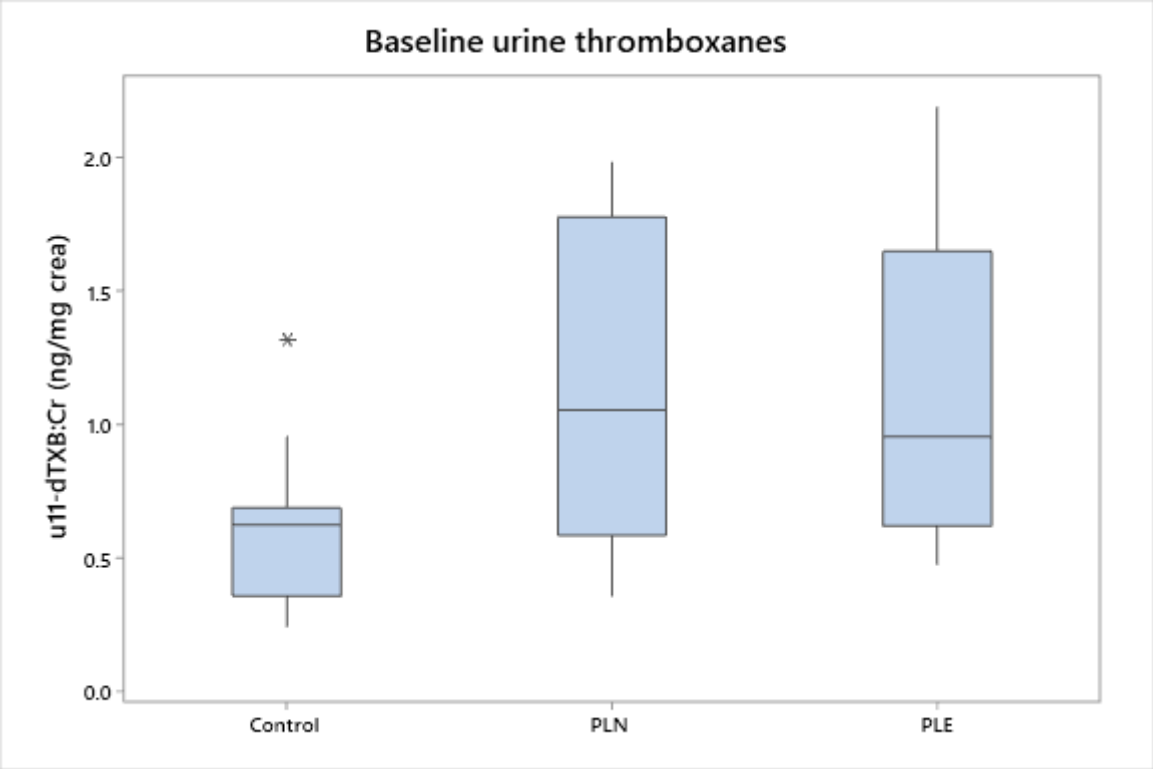
3.2.4 PLN results

The 8 dogs with PLN had u11-dTXB:Cr results ranging from 0.36 to 1.98 ng/mg crea. These values were not normally distributed. The median u11-dTXB:Cr was 1.06 ng/mg crea (SD 0.61), which was not significantly different from the median for the normal dogs (median 0.62 ng/mg crea (SD 0.28, range 0.24-1.32)(p=0.065) (Figure 13, and Figure 5 in comparison to IMHA). A post-hoc power calculation for this data gave β=0.64.

3.2.5 PLE results

The 7 dogs with PLE had u11-dTXB:Cr results ranging from 0.48 to 2.19 ng/mg crea. These values were not normally distributed. The median u11-dTXB:Cr was 0.96 ng/mg crea (SD 0.62), which was significantly higher than the median for the normal dogs (median 0.62 ng/mg crea (SD 0.28, range 0.24-1.32)(p=0.026) (Figure 13, and Figure 5 in comparison to IMHA). A post-hoc power calculation for this data gave β=0.60

Figure 13: Box and whisker plots of u11-dTXB:Cr for dogs with PLN and PLE, compared to control dogs. Boxes represent 75th percentile, median and 25th percentiles. Asterisks represent outliers.



4 Discussion

This study had three main goals; to assess whether u11-dTXB:Cr is increased in dogs with PLN, PLE, and IMHA compared to normal dogs, to look at whether u11-dTXB:Cr correlates with disease severity, outcome, or incidence of thrombosis in IMHA, and to describe the change in u11-dTXB:Cr over the first weeks of treatment in dogs with IMHA. This discussion will first focus on the baseline measures of u11-dTXB:Cr in dogs with the three conditions, before returning to discuss the serial measures in the IMHA dogs at the end.

4.1 Baseline urinary thromboxanes

Firstly, this study has shown that u11-dTXB:Cr is significantly higher at presentation in dogs with both IMHA and PLE than in normal dogs. It was not, however, statistically different between dogs with PLN and normal controls. This may suggest that increased platelet activation is important in the pathogenesis of the prothrombotic state seen in IMHA and PLE, but not in PLN. In human medicine, u11-dTXB:Cr is a well-established marker of in vivo platelet activation. It has been found to be increased in multiple prothrombotic conditions including stroke, myocardial infarction and atherosclerosis (Foegh et al., 1994)(McConnell et al., 2001)(Eikelboom et al., 2002)(Wang et al., 2018). There are multiple sources of thromboxane within the body, including platelets, vascular endothelial cells, and inflammatory cells such as monocytes (Fontana et al., 2014). As such thromboxane production can be increased in many situations, including activation of platelets, vascular endothelial damage or inflammation, and generalised inflammatory states. Numerous studies have shown that thromboxane is a potent activator of platelets, in dogs and people, and so irrespective of the source, increased serum thromboxane levels will lead to increased platelet activation. Additionally, platelet activation is a key step at the point of thrombus formation, irrespective of the driving factors that have led to that point. Thus increased u11-dTXB:Cr may be considered to represent evidence of increased platelet activation, and so potentially a prothrombotic state, in dogs with IMHA and PLE.

Equally, while the group median u11-dTXB:Cr was not increased in the dogs with PLN, there was significant overlap between the range of u11-dTXB:Cr with the control dogs. This overlap with the normal dogs was also present for both the IMHA and PLE group ranges as well. Given that thrombosis does not occur in all individual dogs with these diseases, and so presumably not all individuals will be prothrombotic, it is unsurprising that there is this overlap. To answer the question of whether u11-dTXB:Cr truly is a useful biomarker of thrombotic risk, future studies would need to identify a

reference range for the level in normal patients, and look for any difference in the incidence of thrombosis in groups of patients with increased u11-dTXB:Cr, compared to those with the same disease and normal levels. At this stage, however, the overlapping ranges of u11-dTXB:Cr seen between the diseased and healthy individuals may suggest that either not all diseased individuals are equally prothrombotic, or that different individuals with the same disease have differing degrees of platelet activation.

The below sections explore the specific relevance of the u11-dTXB:Cr findings further in the individual disease states, before looking into some of the general limitations of this study.

4.1.1 IMHA

More specifically for the dogs with IMHA, the theory that this finding of increased baseline u11-dTXB:Cr represents increased platelet activation is consistent with previous studies showing increased platelet activation in these patients. Increased platelet activation has been demonstrated directly via increased platelet P-selectin expression on flow cytometry, increased platelet microparticle levels, and lower mean MPC concentrations in patients with IMHA. There is also evidence of increased intravascular TF expression and high levels of circulating Neutrophil extracellular traps (NETs), both of which are prothrombotic, and can lead to platelet activation in vitro. Given that IMHA is an acute, highly inflammatory disease, it is possible that the measured u11-dTXB:Cr is not solely of platelet origin, but also from endothelial cells due to vasculitis, and from inflammatory leucocytes themselves. While the mechanism of the increased u11-dTXB:Cr in the IMHA dogs was not specifically investigated in the present study, any correlations with known markers of disease severity, markers of inflammation such as WBC and CRP, and with platelet count were assessed. No correlations between any of these variables and u11-dTXB:Cr were found. As such it could be argued that difference in u11-dTXB:Cr between individual dogs with IMHA is not simply reflective of the severity of their IMHA, the severity of the associated inflammation, nor simply a reflector of platelet number. This implies that u11-dTXB:Cr is a measure of an aspect of the pathophysiology in these IMHA patients that cannot currently be accurately assess, and adds weight to the theory that it may be a marker of platelet activation, and so thrombotic risk. In human, as in veterinary medicine, there is an ongoing need for a predictor of thrombosis in patients with autoimmune haemolytic anaemia. Epidemiologic studies have identified a link between active haemolysis and the risk of thrombosis, since VTE occur predominantly during acute flare ups of disease (Audia et al., 2018; Lecouffe-Desprets et al., 2015). Patients with jaundice, and higher white cell counts are also more likely to suffer VTE (Audia et al., 2018). This is similar to the association between jaundice and thrombosis identified in previous studies in dogs (Carr et al., 2002). To date, however, there are still no biomarkers identified with utility in predicting

thrombosis in either the veterinary or human fields. Researchers have investigated free haemoglobin, NO metabolites, and soluble CD163, a marker of macrophage activation, but have not identified differences in these between patients with and without VTE (Audia et al., 2018). To the authors knowledge, thromboxane levels have not been investigated in human patients with AIHA.

4.1.1.1 Association with Survival

This study additionally evaluated, but found no difference between, u11-dTXB:Cr levels in dogs surviving and not surviving to 30 days and 90 days post presentation. The association between u11-dTXB:Cr and survival was chosen for two reasons. Firstly, thrombosis is reported to be a major cause of mortality in IMHA patients but can be very difficult to detect clinically. As such it seemed likely that there would be an association between non-survival and thrombosis, but with non-survival being easier to document than thrombosis. Thus it was hypothesised that if there were an association between u11-dTXB:Cr and thrombosis, there may also be an association between u11-dTXB:Cr and non-survival. The second reason was that currently, while CHAOS score ≥3 has been associated with an increased risk of death, survival outcome in dogs with IMHA still cannot be predicted from data available at clinical presentation. As such, evaluation of the utility of u11-dTXB:Cr as a general predictor of outcome was felt to be worthwhile, irrespective of any association with thrombosis.

There are multiple possible reasons why this study may not have identified an association between u11-dTXB:Cr and survival outcome. Firstly, there are multiple different reasons why dogs with IMHA may not survive. These encompass patient factors such as disease severity, treatment factors such as choice of immunosuppressant or availability of blood transfusion and owner factors such as willingness or financial ability to pursue treatment. While many canine IMHA patients expire naturally, from cardiac or respiratory failure, as with all veterinary studies assessing survival the option of euthanasia is a significant confounding factor. Some patients will be euthanised due to their deteriorating condition alone, however in most the reasons for euthanasia are more complex, involving owner ethical and financial considerations alongside the clinical state and predicted prognosis for the individual patient. Of the 9 non-survivors in this study, 4 were euthanized, of which two had developed severe neurological signs and euthanasia was considered inevitable. The other two, however, were euthanised due to the need for repeated blood transfusion after 11 and 6 days of therapy respectively, and the possibility that they would have survived with a yet longer duration of treatment cannot be excluded. Equally, for those dogs that died naturally, thrombosis is not the only potential cause of death. Of the 5 patients that died, a thrombus (PTE) was suspected in two and cannot be excluded in a third, however the remaining two patients had both developed renal failure and acidosis at the time of death, with no overt suspicion of thrombosis. Equally, in human medicine,

while historically, VTE was reported to be the major cause of death in patients with AIHA, a more recent study found the risk of death did not differ between patients that did and did not suffer from thrombosis during their treatment. The authors speculated that this was due to improvement in the management of patients with VTE, that meant that some patients with VTE were now surviving with treatment (Audia et al., 2018). Equally in dogs, while thrombosis was seen in 80% of patients in post-mortem studies, a previous study assessing for evidence of PTE in IMHA dogs found no correlation between CT angiogram findings consistent with PTE and outcome, with two dogs with confirmed PTE and two with suspected PTE surviving (R. Goggs et al., 2014). While large thrombi that cause impairment of end-organ function may lead to death, particularly in the case of PTE, smaller thrombi may not, and in some cases can be subclinical entirely. As such, while an initial hypothesis was that u11-dTXB:Cr may be associated with survival, one of the reasons it was not may be that there is only limited association between thrombosis itself and survival. As such this finding does not exclude the possibility that u11-dTXB:Cr may be clinically useful in other ways, in particular for investigating platelet activation.

It is also possible that u11-dTXB:Cr was not associated with survival in this population of dogs because of type II error from the small survivor and non-survivor group sizes. As assessing for an association with survival was a secondary aim of the study, a-priori power analysis was not performed for this comparison. Post-hoc power analysis for both the 30 day and 90 day groups, however, showed that these comparisons were significantly underpowered. In addition, this population may not be truly representative of the larger population of dogs with IMHA. This study was performed in a tertiary referral hospital, and on the whole only the sicker end of the severity spectrum of patients present to the hospital for care. As such, to evaluate whether this population is in line with other larger populations of dogs with IMHA reported in the literature, known markers of disease severity were evaluated for correlation with outcome. As in previous studies, baseline CRP, urea, ALT and ASA score were all significantly higher in 30-day non-survivors, with HCT also being significantly lower. Unlike in previous studies, however, there was no significant difference in total white cell count, bilirubin, or CHAOS score. Again, It is possible this is in part due to type II error from the small group sizes once the cases are divided into survivors and non-survivors. Equally, although not statistically significant, the proportion of dogs surviving with CHAOS score <3 surviving was substantially higher than that of those with score >3 (80% vs 47%), raising the possibility the result was statistically non-significant mainly due to type II error. Overall, these findings suggest the IMHA population in this present study is similar to that reported in the literature previously, although again most of the literature regarding IMHA in dogs has come from similar referral centres. Thus it cannot be excluded that there may be a difference in u11-dTXB:Cr between this population and dogs more mildly affected with IMHA. Equally, as most

dogs in this study had increased CRP, fibrinogen, and white cell counts, consistent with inflammation, it is possible no association was found between u11-dTXB:Cr and these parameters because the population did not include any more mildly affected dogs with IMHA, with lower values.

Additional reasons that could contribute to there being no significant difference in u11-dTXB:Cr between survivors and non-survivors include the effect of differences in treatments on outcome. Standard treatment for dogs with IMHA includes the use of immunosuppressive and anti-thrombotic therapy, however there are several different individual drugs within these categories commonly used in the referral hospital (Goggs et al., 2019; Swann et al., 2019). All therapeutic decisions were made by case clinicians, as is normal hospital policy, with no attempt to standardise therapy. Thus dogs had a wide range of immunosuppressive strategies, with varying drug combinations and dosages. This study was not designed to assess the impact of therapeutics on outcome, and so no comparisons of survival between different regimens was attempted, however it is possible that some dogs received treatment regimens that were more effective than others. In particular clinicians will often start dogs perceived as being sicker on multiple immunosuppressives from the outset, rather than starting with a single immunosuppressive. Whether this improves the efficacy of treatment is currently unknown. However differences in treatment such as this may have been a confounding influence on any relationship between baseline u11-dTXB:Cr and survival. As such it would be interesting to further evaluate u11-dTXB:Cr in any future prospective studies assessing the impact of standardised treatments on outcome.

Finally, it is possible that other factors differing between the dogs at baseline had an impact on the u11-dTXB:Cr levels, and masked any association with survival. There was no significant difference in age between the IMHA survival subgroups, nor was there a correlation between age and u11-dTXB:Cr on regression analysis, to suggest that age has a significant impact on u11-dTXB:Cr levels. Associations between u11-dTXB:Cr and breed were not evaluated given the small samples sizes within this study. Several breed-specific differences in haematological and biological parameters are well-known in veterinary medicine, particularly in sighthound breeds, so it is possible that u11-dTXB:Cr may also show some breed-associated variability, and this accounts for the differences in this IMHA population (Zaldívar-López et al., 2011). In addition, most IMHA dogs were already receiving at least one medication at the time of inclusion in this study. It was an inclusion requirement that all dogs were not already receiving anti-thrombotic therapy at the time the urine sample for u11-dTXB:Cr was collected, however for practical reasons patients were only excluded if they had been receiving corticosteroids for over 48h, similar to other veterinary studies of IMHA. However the majority of dogs had received one or more dosages of steroid at the time of inclusion. There is evidence in both human and veterinary medicine, that corticosteroids cause changes to some haemostatic parameters, and

may be prothrombotic (deLaforcade et al., 2019; van Zaane et al., 2010). A recent consensus statement on populations at risk for thrombotic disease in dogs and cats thus came to two conclusions regarding glucocorticoids in dogs. Firstly that corticosteroid administration favours a hypercoagulable state in dogs. And secondly that treatment with corticosteroids may be associated with the development of thrombosis in dogs, in particular those with other risk factors for thrombosis (deLaforcade et al., 2019). In a study evaluating the effects of various immunosuppressives on u11-dTXB:Cr in 8 healthy dogs, prednisolone was found to increase u11-dTXB:Cr in some, but not all of them (Thomason et al., 2018). Overall the impact of prednisolone was very inconsistent, but it does raise the possibility that prior glucocorticoid treatment had an impact on u11-dTXB:Cr levels in dogs in this present study. This may have both impacted on the magnitude of difference between IMHA dogs and normal dogs, and also been a confounding factor on any association between u11-dTXB:Cr and survival outcome.

4.1.1.2 Association with Thrombosis

Baseline u11-dTXB:Cr also did not differ between dogs that did and did not go on to have suspected thrombotic complications. This may suggest that u11-dTXB:Cr is not useful as a marker of thrombotic risk, but may also be due to this comparison being underpowered, as shown by a post-hoc calculation giving the power as only 6.5%. There were also a number of significant limitations around the detection of thrombosis in this study. As outlined in the introduction, detection of thrombi is difficult in both human and veterinary patients. In none of the five patients with a suspicion of thrombosis was the diagnosis confirmed; they were all classified on consistent clinical signs alone. None of the dogs suspected of having CNS thrombosis had confirmatory MRI, nor did those with suspected PTE have CT angiography, as all but one were euthanised or died at the time of suspect thrombosis. Equally, consent for post-mortem was not given for any of the non-surviving patients, either with or without a suspicion of a thrombus at the time of death. This means not only was the clinical suspicion not confirmed in any patient, but that subclinical thrombosis in those who died also cannot be excluded. As there was also limited ongoing screening for thrombosis in the surviving patients, subclinical thrombosis in that population is equally possible. Ideally repeat thoracic and abdominal imaging would have been performed both at fixed serial time-points over the course of treatment, and at any time thrombosis was suspected, however this was not justifiable clinically. It was initially intended to measure FDPs and D-dimers at the time of presentation to look for correlations between the parameters and u11-dTXB:Cr. However clinicians only requested FDPs and D-dimers as part of their clinical work-up in 4 of the 20 included cases, and in no cases was surplus blood collected into sodium citrate tubes for later analysis as per the study request guidelines. As such it was not possible to look for correlations between FDPs, D-dimers and u11-dTXB:Cr. Overall the likely most significant factor

that may have impacted on these results is inaccurate classification of dogs into the thrombus status groups. Further studies with a focus on better detecting thrombi, plus additional measures of hypercoagulability such as TEG would be useful to better establish how u11-dTXB:Cr levels correlate with clinical risk of thrombosis to individual patients.

Aside from these limitations regarding detecting thrombi, there may be other causes for the lack of an association between u11-dTXB:Cr and thrombosis. Firstly it is possible that u11-dTXB:Cr does reflect increased platelet activation, but the physiological relationship between platelet activation and subsequent thrombus formation is not straightforward, with other factors such as blood stasis or hypercoagulability playing a more significant role in whether a thrombus actually forms in dogs with IMHA. Secondly, although there is a large body of research showing that thromboxane A2 causes activation of platelets, in one early study looking specifically at canine platelet function it was found that only 30% of dogs have platelets sensitive to arachidonate, the precursor of thromboxane (Johnson et al., 1979). Further research suggested that responsiveness to thromboxane is inherited as an autosomal recessive trait in dogs (Johnson et al., 1991). As such, it can be speculated that u11-dTXB:Cr might be a marker of thrombotic risk only in that 30% of dogs with platelets sensitive to its effect. In those dogs without platelets sensitive to thromboxane, there may be very little relationship between thromboxane levels and the level of platelet activation, and so also no relationship between u11-dTXB:Cr and the occurrence of thrombosis. Equally, although activated platelets do themselves produce thromboxane, irrespective of what the activating signal is, u11-dTXB:Cr is also derived from other sources of thromboxane such as inflammatory cells and endothelial cells. While in healthy dogs, a number of studies have shown changes in u11-dTXB:Cr associated with alterations in platelet function, it is unknown whether platelets are the main source of thromboxane production in dogs with diseases such as IMHA (Dudley et al., 2013; Thomason et al., 2016, 2018). In people, under normal physiological conditions, between 70 and 80% of serum thromboxane is produced by platelets, with monocytes being the main extra-platelet source (Lellouche et al., 1990). However the proportion of thromboxane produced by monocytes has been shown to be increased in various inflammatory states, in particular by inflammation associated with atherosclerotic plaques (Wang et al., 2018). Whether the similar situation occurs in dogs is unknown, but would be physiologically plausible. As such, changes in u11-dTXB:Cr in dogs with disease may not always result from, or lead to, changes in platelet activation.

This study also did not evaluate the relationship between u11-dTXB:Cr and the time at which a thrombus was suspected. It is known that D-dimer levels show very acute changes at the time thrombosis occurs. In one study of D-dimer pharmacokinetics, there was a sharp peak in their concentration in the first two hours after PTE occurrence, with levels declining back to baseline within

24 hours (Ben et al., 2007). There may be a similar chronological association between u11-dTXB:Cr and thrombosis, with u11-dTXB:Cr levels increasing only very shortly before thrombi occur, as mass platelet activation begins. This study was designed with the primary aim of evaluating whether u11-dTXB:Cr was increased in dogs with IMHA, with evaluating for any association with thrombosis as a secondary aim. As such u11-dTXB:Cr was not serially evaluated more often than every 7-14 days, so any acute changes in u11-dTXB:Cr could have been missed in those patients with suspected thrombi.

Finally, as with evaluating for an association with survival, it is possible that differences in anti-thrombotic therapy between the dogs was a confounding factor on any relationship between their initial u11-dTXB:Cr and whether they subsequently developed thrombi. While all clinicians chose clopidogrel as antithrombotic therapy in the IMHA dogs, a range of different dosages were used, likely largely dictated by tablet size available. This may have variably impacted on the subsequent risk of thrombosis in individual patients, and confounded any relationship with baseline u11-dTXB:Cr.

Overall the finding that u11-dTXB:Cr did not differ in dogs with and without suspected thrombi in this study may indicate it will not be a useful future biomarker of thrombotic risk in dogs with IMHA. However there were a number of limitations to this present study that mean further evaluation of u11-dTXB:Cr alongside additional markers of platelet activation, and with more thorough evaluation for the presence or absence of thrombosis would be required to better evaluate its utility.

4.1.2 PLN

In this study, there was no significant difference in the u11-dTXB:Cr in dogs with PLN compared to the controls. The difference in the medians was similar, however, to that of the PLE dogs, and did approach significance, with a p-value of 0.07. Post-hoc power analysis showed this comparison to be underpowered to detect a statistically significant difference between the groups, with a power of only 64%. As such it is possible that the small case numbers have led to a type 2 error, and inclusion of a larger number of dogs would have revealed a significant difference. In particular, given the range of u11-dTXB:Cr seen in the PLN dogs is wider than that of the normal controls to a similar degree as that of the PLE dogs, it seems likely that there is significant individual animal variation in thromboxane levels. Unfortunately, despite the study covering a two-year period, fewer dogs with PLN were recruited to the study than was anticipated. In part this was due to a diagnosis of PLN often being reached from test results received after patients had already left the hospital, so that fresh urine samples were not available for inclusion into the study.

It is also possible no difference in u11-dTXB:Cr between the groups was found because the pathogenesis of thrombosis in PLN is different to that in PLE and IMHA, and platelet activation does

not play as significant a role in the mechanisms of thrombotic risk, or only occurs later in the risk period when active clot formation has started. In the human field, the main abnormalities thought to contribute to thrombosis in nephrotic syndrome are to do with alterations to the coagulation system, including loss of anti-coagulant molecules in the urine. However evidence of increased platelet activation has also been found in numerous studies. It has additionally been shown that u11-dTXB:Cr levels are higher in people with new-onset or relapsing nephrotic syndrome compared to healthy controls. Taken together this suggests that a type 2 error may be likely in this present study, and including a larger number of PLN dogs may have found a significant difference. So far in dogs, there has been very little work done looking at the role of platelet activation in the prothrombotic state associated with PLN. The majority of research has focused either on the contribution of anti-thrombin, or on measures of hypercoaguability. As such very little is known about whether platelet activation may be similarly increased in dogs with PLN as it Is in people with nephrotic syndrome. Work in dogs with uraemia due to CKD has, however, identified evidence of platelet activation, in the form of increased surface expression of GPIb, αIIbβ3, and P-selectin (Dudley et al., 2017). As such further evaluation of u11-dTXB:Cr in a larger population of dogs with PLN, including differentiation into groups of uraemic and non-uraemic animals would be useful to better clarify the situation.

4.1.3 PLE

Dogs with PLE also had higher u11-dTXB:Cr than normal dogs in this study. Again, while this thromboxane may be from various sources, the hypothesis was that increased levels would lead to increased platelet activation, and so a higher risk of thrombus formation. Other than this current study, to the authors knowledge there have been no previous studies looking specifically at the role of platelet activation in canine PLE. The only prospective study evaluating TEG in canine PLE did not specifically evaluate the contribution of platelet factors, as has been done by some authors for canine IMHA (Goodwin et al., 2011; Hamzianpour and Chan, 2016). In the human field, there is evidence that increased platelet reactivity plays a role in thromboembolism in both Crohn's disease and IBD. Studies have found evidence of increased platelet surface expression of P-selectin, GP53 and CD40, all of which are prothrombotic, and increased serum levels of β-thromboglobulin, CD40 and platelet factor 4, which are released only by activated platelets (Danese et al., 2004; Senchenkova et al., 2015). It has also been shown that human IBD patients have higher u11-dTXB:Cr levels than healthy controls, and patients with active disease have higher levels than IBD patients in remission. Interestingly the magnitude of the increase in u11-dTXB:Cr seen in the IBD patients in the human study (median 0.76 ng/mg crea, IQR 0.49-1.34) is very similar to that seen in the PLE dogs in this present study (Di Sabatino

et al., 2016). Although this study only had a small population of dogs, the finding of increased u11-dTXB:Cr is in agreement with findings in the human field, and suggests that increased platelet activation is likewise important in the pathogenesis of PLE in canine patients. Whether this increase in u11-dTXB:Cr correlates with an increased risk of thrombosis remains to be determined.

Like with the IMHA dogs, the source of this thromboxane was not specifically investigated in this study, but is likely multifactorial, coming from activated platelets, inflammatory cells, and endothelial cells. In humans, immunostaining of colonic biopsy samples from people with IBD showed increased thromboxane synthase levels in the lamina propria layer compared to healthy controls. They also found higher staining in those patients with active disease than in those with inactive disease, which suggests the gut may be the source of the higher u11-dTXB:Cr seen in the previous human study (Carty et al., 2002). Thromboxane synthase staining was increased both in the lamina propria macrophages, and in other cell types, speculated to be lymphocytes or epithelial cells. It has been suggested that increased thromboxane levels within the GI microcirculation play a role in the pathogenesis of IBD, by promoting formation of microvascular thrombosis within the gut wall, leading to micro-infarction and promoting inflammation (Dhillon et al., 1992; Rampton and Collins, 1993) . As such, thromboxane synthase inhibitors have also been investigated as therapeutics for IBD and Crohn's disease and have shown promising results decreasing urinary thromboxane levels and disease activity in some, but not all, initial in vivo and in vitro studies (Casellas et al., 1995; Howes et al., 2007; Tytgat et al., 2002). Should thromboxane production also be increased in a wider population of dogs with PLE or IBD, and be similarly increased locally within the GI tract, this may also represent a novel therapeutic target both for disease severity within the gut, as well as for systemic thrombosis risk modulation. A limitation of this present study is that no correlations between u11-dTXB:Cr and markers of disease severity such as as CIBDAI or CCECAI were performed. As such whether the dogs with the highest u11-dTXB:Cr had the highest disease activity, as is the case in people with IBD is unknown (Di Sabatino et al., 2016).

Overall, further studies looking at u11-dTXB:Cr in a wider population of dogs with PLE and IBD, ideally alongside measures of GI thromboxane synthesis and disease activity scores such as CIBDAI or CCECAI, would be interesting. It may also be possible to further investigate whether u11-dTXB:Cr can predict thrombosis in a larger study population. To the authors knowledge, at present there are no studies in people investigating whether higher u11-dTXB:Cr correlates with clinical incidence of thrombosis.

4.2 Serial measures in IMHA

The final part of this study was measuring u11-dTXB:Cr over time in the surviving dogs with IMHA. The key finding was that median u11-dTXB:Cr was still significantly higher for the surviving dogs as a group than for the healthy control dogs at all time points up to 6 weeks into treatment. Additionally, at each time point, the majority of individual surviving dogs had u11-dTXB:Cr that was higher than the highest normal dog value (1.32ng/mg crea). This suggests that there is still a drive for platelet activation in at least some patients with IMHA by week 6 of treatment. This may mean the risk period for thrombosis extends for longer than the period of acute illness. At present, while antithrombotic therapy is considered part of the standard of care for dogs with IMHA, there is no consensus as to how long this therapy should be continued (Blais et al., 2019; Swann et al., 2019). At present ACVIM guidelines suggest thromboprophylaxis for at least the first two weeks following diagnosis, and for as long as the dog is receiving prednisolone. However there is only a very limited evidence base behind this suggestion. In humans with autoimmune anaemia, the risk of VTE has been found to be high for the entire first 3 months following diagnosis (Audia et al., 2018; Yusuf et al., 2015). The finding in the present study that some dogs still have increased u11-dTXB:Cr by as late as day 43 of treatment adds support to the hypothesis that some dogs will still have increased thrombotic risk beyond the initial two week period.

A complicating factor in interpreting the serial u11-dTXB:Cr results is the impact of medications. Unlike at baseline, where patients had received very limited therapeutics prior to presentation, the dogs were on a wide variety of medications at the time of recheck examinations. All the dogs were receiving glucocorticoids and clopidogrel, at a range of dosages, and three were additionally on ciclosporine. Other prescribed medications included omeprazole, antibiotics for secondary urinary and GI infections, and faecal binding agents amongst others. Of these, ciclosporine and glucocorticoids have both been found to increase u11-dTXB:Cr levels in healthy dogs (Thomason et al., 2018). As such it is possible that the persistent increases in u11-dTXB:Cr are due to medication effects, rather than being due to the underlying disease. Conversely, some anti-platelet drugs are well known to reduce thromboxane production by platelets. Aspirin does this via direct inhibition of cyclooxygenase, and has been shown to reduce u11-dTXB:Cr in healthy dogs at a variety of dosages (McLewee et al., 2018; Thomason et al., 2016). Mechanistically, clopidogrel might not be expected to affect thromboxane production, as it acts via surface P2Y12 receptor antagonism rather than through direct COX inhibition, and there are multiple alternative platelet activation pathways besides that of P2Y12 that can lead to induction of COX activity and so thromboxane production. Although at present there are no veterinary studies assessing the impact of clopidogrel on u11-dTXB:Cr, studies in healthy people showed that clopidogrel

can cause a similar reduction in u11-dTXB:Cr to aspirin (Armstrong et al., 2010). Thus overall it could be expected that the varying medications the dogs in this study were receiving might have a range of different impacts on their overall thromboxane production. Whether medication-induced increases in thromboxane alone would lead to a prothrombotic state and increase the risk of thrombosis is unknown, however there is a body of evidence showing that high-dose prednisolone is prothrombotic (van Zaane et al., 2010). As argued previously, however, high thromboxane would be expected to lead to increased platelet activation, irrespective of the source. Given the risk of thrombosis in individual patients will be due to a combination of effects of the underlying disease, plus any prothrombotic and anti-thrombotic medications being given, it may be a strength of u11-dTXB:Cr as a biomarker that it is also affected by all of these factors. Exemplifying this, in the human field, u11-dTXB:Cr has been found to correlate with the ongoing risk of thrombosis in patients already on aspirin anti-thrombotic therapy following a previous acute coronary event (Eikelboom et al., 2002). As such the sustained increase in u11-dTXB:Cr found in dogs in this study is interesting, and warrants further investigation.

4.3 Limitations

As with all clinical research, there were a number of limitations to this study. Some of these, such as the difficulty in detecting thrombosis in the IMHA dogs, have already been expanded on in the relevant sections above. For both the PLE and PLN baseline measures, the aim was to recruit more than seven and eight dogs respectively, but there were fewer cases of each disease presented to the clinic during the study period than had been anticipated. Additionally, cases were often only identified as having these diseases on receipt of test results returned after the dogs had left the clinic, which meant the opportunity for recruitment and urine sample collection had been missed. In a number of cases further follow-up or dispensing of medications was performed by the referring veterinary surgeon, due to geographical distance from the hospital so there was also no opportunity to collect urine samples at a later date. These limitations were unavoidable given the nature of referral practice, but it did mean the case numbers for both PLE and PLN were smaller than had been hoped. For PLN this small sample size may have contributed to this study not finding a significant difference in baseline u11-dTXB:Cr from that of normal dogs. Being a referral centre may have also skewed the population of IMHA dogs included in this study towards those patients requiring more intensive care, so it must also be borne in mind that the finding of increased u11-dTXB:Cr may not hold true in less severely affected IMHA dogs.

An additional limitation was that while u11-dTXB:Cr was measured in a population of healthy dogs of similar size to the IMHA population, this study did not set out to quantify a normal reference range

for canine u11-dTXB:Cr. According to current ASVCP guidelines, an ideal population size of at least 120 individuals is needed for reliable calculation of a reference interval. Populations of as low as 20 samples can also be used under specific circumstances, however wider 90% confidence intervals around the reference limits must be used, and additional samples must be collected if the CI exceeds 0.2 times the calculated reference interval. As such this population of 16 healthy dogs (reduced to 15 after removal of an outlier), would not be appropriate for generation of a reference interval (Friedrichs et al., 2012). The range of thromboxane seen in the healthy dogs was, however, very similar to that seen in other studies of healthy dogs where the unit u11-dTXB:Cr was measured in was reported. In the biggest study including 20 healthy dogs, the median u11-dTXB:Cr was 0.38 ng/mg crea, with a range of 0.15-1.13 ng/mg crea (Hoh et al., 2011). However further work to recruit additional healthy dogs and measure u11-dTXB:Cr in another 20-30 animals (to allow for outliers) would be worthwhile. This would also help to better clarify the relevance of the u11-dTXB:Cr in individual animals. In all 3 diseases there was an overlap between the range of u11-dTXB:Cr in diseased and healthy individuals. This may suggest that not all animals with these diseases have equal degrees of platelet activation, and so are not all at equal risk of thrombosis. While the ideal situation would be to have a clinical cut-off above which the risk of thrombosis is considered significant, establishing which animals have any degree of increased risk would be a useful first step.

The final limitation to this work that ought to be highlighted, is that it was not possible to assess other markers of platelet activation or prothrombotic phenotype in conjunction with u11-dTXB:Cr. It was expected that a larger proportion of clinicians would measure D-dimers in IMHA patients at baseline than transpired to be true, meaning it was not possible to look for any correlation with u11-dTXB:Cr. Equally, while a number of studies have identified both hyper- and hypo-coagulability in IMHA using viscoelastic tests such as TEG, and these machines are increasingly available in referral institutions, this methodology was not available for this study. Given this study has identified increased u11-dTXB:Cr both at baseline and over the first 6 weeks of treatment in dogs with IMHA, investigators planning future studies looking at aspects of haemostasis in these patients could consider storing urine samples for concurrent u11-dTXB:Cr measurement.

4.4 Final conclusions

The studies described in this thesis have added to our knowledge of urinary thromboxanes as potential markers of platelet activation in dogs with three common prothrombotic conditions. While it remains to be seen if u11-dTXB:Cr does correlate with the clinical risk of thrombosis, this study has explored some difficulties surrounding accurate thrombus detection that will have to be considered in any

future studies aiming to quantify this. The big advantage of u11-dTXB:Cr as a urine biomarker compared to other measures of platelet activation is that it is non-invasive to the patient to collect samples, and since it is stable at room temperature for up to 6 days does not rely on point-of-care testing or rapid transport to the laboratory. Urine markers of platelet activation also avoid the complications of in vitro platelet activation either during or after blood sampling that can affect blood-based parameters such as platelet surface molecule expression. For now, u11-dTXB:Cr may represent a promising new biomarker of platelet activation, which is increased in dogs with both IMHA and PLE.

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6 Appendix 1

Supplemental tables (Appendix 1, Tables 1-4) of basic case data, baseline clinicopathological test results, treatment information and outcomes for 20 dogs with primary IMHA.

Appendix 1 Table 1: Basic case data and IMHA diagnostic criteria for 20 individual dogs with primary IMHA

Basic case data					IMHA diagnostic criteria					
Study number	Breed	Age (years)	Weight (kg)	Sex	In-saline agglutination	Direct coombs	Spherocytes	Haemoglobinuria	Hyper-bilirubinaemia (>10umol/l)	ACVIM consensus statement diagnosis category
IMHA 001	Border Collie	9	19	Fe	positive (macroscopic)	n/a	positive	positive	no	diagnostic
IMHA 002	Labrador	11	31.8	Fn	positive (microscopic)	positive 1/64	positive	negative	yes	diagnostic
IMHA 003	Springer	10	26.5	Me	positive (macroscopic)	positive 1/128	negative	positive	yes	diagnostic
IMHA 004	Cocker spaniel	5	18	Fn	positive (macroscopic)	positive 1/128	equivocal	negative	yes	diagnostic
IMHA 005	Whippet	10	10	Fe	positive (macroscopic)	n/a	positive	positive	yes	diagnostic
IMHA 006	Bichon frise	6	8.6	Fe	positive (microscopic)	n/a	positive	positive	yes	diagnostic
IMHA 007	Cairn terrier	12	10.7	Mn	positive (macroscopic)	positive 1/128	positive	negative	no	supportive
IMHA 008	Cocker spaniel	4	11.5	Fe	positive (macroscopic)	positive 1/128	positive	positive	yes	diagnostic
IMHA 009	Irish Setter	8	30.3	Me	positive (microscopic)	positive 1/128	positive	positive	yes	diagnostic
IMHA 010	Cross breed	5	19.9	Fn	positive	n/a	positive	positive	yes	diagnostic
IMHA 011	French bulldog	6	12	Fn	positive (macroscopic)	n/a	positive	positive	yes	diagnostic
IMHA 012	Cockapoo	7	12.4	Fn	positive (macroscopic)	n/a	positive	positive	yes	diagnostic
IMHA 013	Miniature Schnauzer	12	9.9	Fn	positive (macroscopic)	n/a	positive	negative	no	supportive
IMHA 014	Jack Russel	9	6.4	Fn	positive (macroscopic)	positive 1/128	positive	positive	yes	diagnostic
IMHA 015	Crossbreed	9	13.2	Fn	positive (microscopic)	positive 1/8	negative	negative	no	supportive
IMHA 017	Border Collie	10	23	Fn	positive	n/a	positive	positive	yes	diagnostic
IMHA 020	Cocker spaniel	6	19.3	Fn	positive (macroscopic)	n/a	positive	positive	yes	diagnostic
IMHA 021	Labradoodle	2	25	Mn	positive (macroscopic)	n/a	positive	positive	yes	diagnostic
IMHA 022	Springer spaniel	5	20.5	Fn	positive (macroscopic)	positive 1/64	positive	positive	yes	diagnostic
IMHA 023	Pomeranian	4	4	Mn	positive (macroscopic)	n/a	positive	positive	yes	diagnostic

Abbreviations: IMHA, Immune mediated haemolytic anaemia, Fn, female neutered; Mn, male neutered; Me, male entire; Fe female entire

Appendix 1 Table 2: Selected clinicopathological data, ASA and CHAOS scores for 20 individual dogs with primary IMHA

Study number	Day 1 Scores		Selected Day 1 Haematology				Selected Day 1 Biochemistry						Selected Day 1 Other		
	ASA Score	CHAOS score	HCT (%)	reticulocyte count ($x10^{12}$/l)	WBC ($x10^9$/l)	PLT count ($x10^9$/l)	Urea (mmol/l)	creatinine (umol/l)	total bilirubin (umol/l)	ALT (IU/L)	TP (g/l)	albumin (g/l)	CRP (mg/l)	fibrinogen (mg/dl)	urine 11-dTXB:Cr (ng/mg crea)
IMHA 001	4	4	11.4	0.306	24.35	499	15.4	61	0		82	36	120.6	424	2.69
IMHA 002	3	4	19.6	0.2209	14.44	190	5	94	19	26	72	36	164.3	540	4.03
IMHA 003	4	6	11.6	0.061	22.94	*125	12.1	59	143	25	69	28	121.3	653	2.2
IMHA 004	3	1	25.5	0.1089	9.62	*125	7.8	51	59	36	76	37			1.57
IMHA 005	3	4	19.9	0.143	35.25	401	5.8	64	16	30	65	22	152.8	718	0.97
IMHA 006	3	2	16.2	0.199	20.17	*350	7	40	19	32	78	34	230.7	777	4.6
IMHA 007	2	3	17	0.119	19.02	269	8.9	64	7	89	73	33	187.7	634	1.4
IMHA 008	4	4	13.2	0.176	26.78	153	15.8	35	352	103	93	42	274	718	5.91
IMHA 009	4	6	10.3		51.15	285	27	253	667		72	34	229.9	572	3.48
IMHA 010	4	3	14.2	0.016	27.66	343	12.5	69	121	56	67	30		718	4.29
IMHA 011	4	4	11.3	0.0891	14.91	*125	9.1	70	>477	217	90	40			2.8
IMHA 012	3	7	12.3		39.55	846	12.9	53	87	36	70	28		587	1.1
IMHA 013	3	3	22.9	0.078	13.67	333	6.1	48	4	72	82	34			8.37
IMHA 014	4	4	19.5	0.156	15.92	*350	8.2	59	18	1321	66	27	348.1	456	1.71
IMHA 015	3	3	14.3	0.307	25.36	*750	5.3	39	6	50	57	30	40.9	368	25.36
IMHA 017	5	6	11				23.2	191	357	171	95	49			5.61
IMHA 020	3	1	15.6	0.076	18.54	385	112	37	46		89	38		712	7.66
IMHA 021	3	2	18.2	0.2071	31.09	101		60	27		73	35			5.8
IMHA 022	4	3	17	0.116	12.75	181	16.2	113	294	2704	59	31	286.7	388	6.28
IMHA 023	3	1	16	0.156	25.13	*350	10	62	30	237	68	31	110	616	0.83

Abbreviations: WBC, total white blood cell count; PLT, platelet; CRP, c-reactive protein; TP, Total protein

Notes: platelet counts marked * are estimated values based on blood smear examination comments from a clinical pathologist as follows; 'adequate' = 125, 'reference' = 350, 'increased' = 750

Appendix 1 Table 3: Outcome data for 20 individual dogs with primary IMHA

Study number	Day of death (post diagnosis)	Cause of death (IMHA vs other)	Follow-up day last known alive (post diagnosis)	Survival status at 30 days	Survival status at 90 days	Thrombus status
IMHA 001	6	IMHA		non-survivor	non-survivor	not suspected
IMHA 002	316	other		survivor	survivor	not suspected
IMHA 003	58	IMHA relapse		survivor	non-survivor	suspected
IMHA 004	n/a		654	survivor	survivor	not suspected
IMHA 005	n/a		732	survivor	survivor	not suspected
IMHA 006	11	IMHA		non-survivor	non-survivor	not suspected
IMHA 007	473	other		survivor	survivor	not suspected
IMHA 008	6	IMHA		non-survivor	non-survivor	not suspected
IMHA 009	2	IMHA		non-survivor	non-survivor	not suspected
IMHA 010	3	IMHA		non-survivor	non-survivor	suspected
IMHA 011	2	IMHA		non-survivor	non-survivor	suspected
IMHA 012	n/a		395	survivor	survivor	not suspected
IMHA 013	n/a		319	survivor	survivor	not suspected
IMHA 014	6	IMHA		non-survivor	non-survivor	not suspected
IMHA 015	68	unknown		survivor	non-survivor	not suspected
IMHA 017	1	IMHA		non-survivor	non-survivor	suspected
IMHA 020	n/a		232	survivor	survivor	not suspected
IMHA 021	120	IMHA relapse		survivor	survivor	not suspected
IMHA 022	1	IMHA		non-survivor	non-survivor	suspected
IMHA 023	n/a		163	survivor	survivor	not suspected

Abbreviations: IMHA, immune mediated haemolytic anaemia

Appendix 1 Table 4: Treatment information for 20 dogs with primary IMHA

Study number	Initial treatment data									Subsequently started immunosuppressives		
	Transfusion	AT drug	AT dose (mg/kg/day)	Initial IS drug A	Initial IS drug A dose (mg/kg/day)	Initial IS drug B	Initial IS drug B dose (mg/kg/day)	Initial IS drug C	Initial IS drug C dose (mg/kg/day)	Additional IS drug D	Additional IS drug D dose (mg/kg/day)	Day additional IS drug D added
IMHA 001	pRBC	clopidogrel	2.0	dex	0.30	myco	13.2					
IMHA 002	none	clopidogrel	2.4	dex	0.31	myco	7.9					
IMHA 003	pRBC	clopidogrel	2.8	dex	0.30	myco	10.0	human IgG	0.5g/kg (one-off)	cyclo (replaced myco)	5	6
IMHA 004	pRBC	clopidogrel	2.1	pred	1.94	myco	13.9					
IMHA 005	pRBC	clopidogrel	3.8	pred	2.00	cyclo	5.0					
IMHA 006	pRBC	clopidogrel	2.2	dex	0.23	cyclo	16.3					
IMHA 007	none	clopidogrel	1.7	dex	0.30					myco	9.3	6
IMHA 008	pRBC	clopidogrel	1.6	dex	0.30	myco	10.0					
IMHA 009	pRBC	clopidogrel	1.2	dex	0.30	myco	10.0					
IMHA 010	pRBC	clopidogrel	1.9	dex	0.18	myco	12.1					
IMHA 011	pRBC	clopidogrel	3.1	dex	0.28	aza	2.1					
IMHA 012	pRBC	clopidogrel	1.5	dex	0.32	myco	8.1			cyclo	4	9
IMHA 013	none	clopidogrel	1.9	pred	2.02	myco	20.2					
IMHA 014	pRBC	clopidogrel	2.0	dex	0.30	cyclo	7.8					
IMHA 015	pRBC	clopidogrel	2.8	dex	0.07	myco	24.2					
IMHA 017	none	clopidogrel	1.6	dex	0.52							
IMHA 020	pRBC	clopidogrel	1.0	dex	0.31	myco	13.0					
IMHA 021	none	clopidogrel	3.0	dex	0.50	aza	2.0					
IMHA 022	pRBC	clopidogrel	1.8	dex	0.60	myco	19.5					
IMHA 023	pRBC	clopidogrel	4.7	dex	0.30	cyclo	10.0					

Abbreviations: IMHA, Immune mediated haemolytic anaemia; IS, immunosuppressive; pRBC, packed red blood cells; IgG, immunoglobulin; dex, dexamethasone; pred, prednisolone; myco, mycophenolate; cyclo, cyclosporine; aza, azathioprine