Investigation of the clinical safety and efficacy of lyophilized platelets in the treatment of bleeding associated with thrombocytopenia in dogs

Masters in Veterinary Research

Edinburgh University: 01.10.2018-01.10.2020

Jenny Walton BVM&S MRCVS

Primary Supervisor -Professor Richard Mellanby BSc BVMS PhD DSAM DipECVIM-CA FRCVS

Secondary supervisor - Simon Tappin MA VetMB CertSAM DipECVIM-CA FRCVS

<u>Clinical Trial approval</u>

VMD ATC: 51028/4000

<u>Welfare and Ethical Approval</u>:

Edinburgh University (VERC) – 78.19

Royal Vet College London (CRERB): URN 2019 1880-3

<u>Clinical Trial sponsor</u>:

Anne S. Hale DVM Bodevet Inc

Contents

Masters Abstract

Introduction

Platelets (thrombocytes) are an essential part of the mammalian haemostasis system. Platelets localise to sites of injury, adhere, activate and aggregate in a process termed primary haemostasis. Thrombocytopenia (a reduction in circulating platelets), can lead to an increased risk of bleeding and is an important cause of morbidity and mortality in dogs. Treatments for this condition are suboptimal and there is an unmet clinical need for superior therapies. Lyophilized platelet preparation involves freeze drying platelets that can be rehydrated and administered into patients intravenously. Despite the potential of lyophilized platelet products to improve the treatment of canine thrombocytopenia, the clinical efficacy of this product is not yet fully understood.

Aims

The aim of this MSc was to undertake a veterinary clinical trial to examine the safety and clinical efficacy of lyophilized platelet products in the treatment of canine thrombocytopenia, using Good Laboratory Practice (GLP) standards utilized in human medical research.

Methods

The study was a prospective, multicentre, randomized, blinded trial comparing the administration of test (lyophilized platelets within a buffer) solution versus control (lyophilized buffer) solution, alongside medical treatment for underlying disease. Trial patients were evaluated over a 24-hour period after enrolment and followed up for 14 days. Nine UK veterinary centres enrolled dogs from July 2019 to July 2020 (ongoing) with a platelet count of $<50 \times 10^9$/l and visible signs of bleeding. Aetiology of thrombocytopenia was not restricted, so long as study inclusion and exclusion criteria were met. The study was designed to evaluate for superiority between groups in primary endpoints and to analyse

secondary endpoints. A power calculation determined that the study should enrol 40 patients, 20 to treatment and 20 to control, which represents 80% power to obtain a statistical difference between treatment groups at the 5% (p=0.05) significance level and takes into account up to 4 dropouts per group.

Results

Within the MSc timeline, the study was designed and 20 dogs were enrolled, including 11 in the test group and 9 in the control group. This included 15 females and 5 males, mean age 6 years. A diagnosis of Immune Thrombocytopenia (ITP) was made in 19 of 20 cases. Interim analysis determined there were no significant differences between groups for the clinical endpoints. Three dogs, including two in the control group and one in the treatment group died within the 24-hour study period. Mean hospitalisation times in the test group was 4 (1-7) days and in the control group 5 (2-8) days. There were no adverse events associated with trial solution administration.

Discussion and conclusions

This study demonstrated the feasibility of undertaking an ambitious, multicentre study to assess the efficacy and safety of a novel therapy for a challenging clinical disorder. Whilst results are from an interim analysis, they reveal that lyophilized platelet administration was not related to any incidence of adverse event. No evidence was found that the novel therapy worsens clinical outcome. Additional recruitment will further define clinical safety and efficacy.

Acknowledgements

Clinical recruitment team leads and masters advisors

Walton JE BVM&S MRCVS (JW)

Hale AS DVM (ASH)

Adamantos S BVSc CertVA DipACVECC DipECVECC MRCVS (SA)

Boiron L MSC DVM DAVECC DipACVECC MRCVS (LB)

Breheny C BVM&S DipECVIM-CA MRCVS (CB)

DiBella A Cert SAM DipECVIM-CA MRCVS (AB)

Holmes A BVSc DipECVIM-CA MRCVS (AH)

Humm K MA VetMB MSc Cert VA DipACVECC DipECVECC FHEA MRCVS (KH)

Ibarrola P DSAM DipECVIM-CA MRCVS (PI)

Lewis DH MA VetMB CVA DipACVECC DEVECC MRCVS (DL)

Walton K BVM&S MRCVS (KW)

Chase-Topping M BSc (Hons) MSc PhD (MCT)

Mellanby R BSc BVMS PhD DSAM DipECVIM-CA FRCVS (RM)

Tappin S MA VetMB CertSAM DipECVIM-CA FRCVS (ST)

Institutional affiliations

JE Walton[1] , AS Hale[1], R Mellanby [2] S Adamantos[3], L Boiron[4], Breheny C[2], A DiBella[5], A Holmes[3], Humm K[6], P Ibarrola[7], DH Lewis[8], K Walton[9], M Chase-Topping[2], S Tappin[10]

[1] Bodevet, 9210 Corporate Boulevard, Ste 310, Rockville, Maryland 20850

[2] Royal (Dick) School of Veterinary Studies, Easter Bush Campus, Roslin, Edinburgh EH25 9RG

[3] Paragon Veterinary Referrals, Paragon Business Village, Red Hall Crescent, Wakefield, WF1 2DF.

[4] Willows Veterinary Centre and referral service, Highlands Road, Solihull, West Midlands, B90 4NH

[5] Southern Counties Veterinary Specialists, Unit 6, Forest Corner Farm, Hangersley, Ringwood, Hampshire, BH24 3JW

[6] Queen Mary Hospital for Animals, Hawkshead Lane, North Mymms, London, AL9 7TA

[7] Davies Veterinary Specialists, Manor Farm Business Park, Higham Gobion, SG5 3HR

[8] Glasgow Vets Now Emergency Hospital, 123-145 North Street, Glasgow, G3 7DA

[9] Washington Vets4Pets, 66 Blue House Lane, Washington, Tyne and Wear, NE37 2TA

[10] Dick White Referrals, Station Farm, London Road, Six Mile Bottom, Suffolk, CB8 0UH

Masters contribution statement:

JW is the study lead, designed the study and authored the thesis. LB,PI,DH,KW, AD and CB recruited patients and collected data. SA,AH,KH and ST were clinical leads for patient recruitment. MCT and JW performed the statistical analysis. RM and ST acted as student supervisors throughout the masters project. ASH assisted with study design, provided editorial guidance on the thesis and acted as the industry sponsor liaison.

Sources of support:

This work was supported as a masters in veterinary research by Edinburgh University. The study lead was supported for the period of study by BodeVet Inc. Clinical centres were supported by Bodevet Inc by donation of lyophilized platelet and control solutions and funding for endpoint laboratory analyses to cover the cost of compulsory testing (credited to the client) for the 24 hour study period.

Conflict of interest statement:

JW has received financial support from BodeVet Inc. for the duration of the masters project. ASH is an employee and shareholder of BodeVet Inc.

Acknowledgement: To Andy Brown, a friend and a driving force in making this study a reality.

Abbreviations

AAALAC - Assessment and accreditation of laboratory animal care

ADAMSTS13 - a disintegrin and metalloproteinase TS13

AE - Adverse events

AABB - American Association of Blood Banks

ATC - Animal Test Certificate

APC - Antiphospholipid syndrome

APC - Apheresis Platelet Concentrate

BET - Bacterial Endotoxin test

BLS - Bleeding score

BSA - Body Surface Area

BSAVA - British Small Animal Veterinary Association

BC - Buffy coat

CD – cluster of differentiation molecules - cell surface markers

CRI - Constant rate infusion

DOGiBAT - Daily canine bleeding assessment tool

PLADO - Determination of the optimal prophylactic platelet dose strategy to prevent bleeding in thrombocytopenic patients trial

DMSO - Dimethyl sulfoxide

DIC - Disseminated intravascular coagulation

DEA - Dog Erythrocyte Antigen

GP – Glycoprotein

GLP - Good laboratory practice

GMP - Good manufacturing practice

HBOC - hemoglobin Oxygen Carrier

IBLS - human ITP bleeding scale

HLA - Human leucocyte antigen

HPA - Human Platelet antigen

IMHA – Immune-Mediated haemolytic anaemia

IMT- Immune-mediated thrombocytopenia

ITP- Immune thrombocytopenia

IL - Interleukins

ICD - International Statistical Classification of Diseases and Related Health Problems

ICH - intracranial haemorrhage

IVIG - Intravenous Immunoglobulin

LAL - Limulus amebocyte lysate

LCP - Lyophilized canine platelets

NHS BT - National Health Service Blood and Transplant

NZWR - New Zealand White Rabbit

NFABB – Non-food animal blood bank

NHP – Non-human primates

PBBuk - Pet Blood Bank UK

PS - phosphatidylserine

PAS - Platelet additive solution

PRP - Platelet rich plasma

Rh - Rhesus

RCVS - Royal College of Veterinary Surgeons

SOP - Standard operating procedures

SToP - Strategies for Transfusion of platelets - SToP randomized controlled trial 2009

TPO - Thrombopoeitin

TTP - Thrombotic thrombocytopenic purpura

TF - Tissue factor

TXA - Tranexamic acid

TTI - Transfusion transmitted infection

TREATT - Trial to evaluAte tranexamic acid therapy in thrombocytopenia

FDA - U.S Food and Drug Administration

UK - United Kingdom

US - United States

VMD - Veterinary Medicines Directorate

vWF - von Willebrand Factor

WHO - World Health Organisation

Literature review

Platelet pathophysiology

Platelets are formed in the haematopoietic tissues, primarily in the bone marrow but also within the lung and peripheral bloodstream. They develop from multipotential progenitor cells called megakaryocytes. This process is considered to be predominantly regulated by the cytokine Thrombopoeitin (TPO),[1] acting alongside other factors such as interleukins (IL) 3,6 and 11. Megakaryocyte cells are large cells that assemble the components of platelets within them. Then, through a process of pseudopodia and evagination, platelets bud off from the parent megakaryocyte as proplatelets.[2] These proplatelets then mature into platelets. Each megakaryocyte has the capacity to generate thousands of platelets.

Platelets are anucleate, so are not considered complete "cells" but rather buds of cellular cytoplasm. Mature circulating platelets are discoid in shape, due to a filamentous microtubular inner membrane. Mammalian platelets are a matter of a few micrometres in diameter, with some variation between species. Variation can be in size but also in mass and the two do not necessarily correlate.[3] Dog platelets are slightly smaller than human platelets when comparing mean platelet volume.[3,4]

The platelet has a bilayer outer membrane ("cytoskeleton") which is predominantly made up of phospholipids. There are transmembrane glycoproteins that span the bilayer membrane, and there is also an array of peripheral proteins on the external surface. The transmembrane glycoproteins frequently act as platelet receptors. Two notable platelet surface receptors involved with the key platelet activity of adhesion and aggregation are the leucine rich glycoprotein (GP) Ib -IX-V which initially binds to immobilised von Willebrand Factor (vWF) within damaged endothelial vessel walls and Integrin GP IIb-IIIa (CD41-CD61) that once expressed during activation binds to fibrinogen.

The bilayer membrane surrounds the central area of the platelet which contains invaginating portions of the external membrane known as the open canalicular system, membrane complexes of the dense tubular system, a filamentous microtubular inner membrane (involved in shape conservation and change depending on activation), and then free within the cytoplasm are organelles, including granules (alpha and dense granules), mitochondria, lysosomes and glycogen.

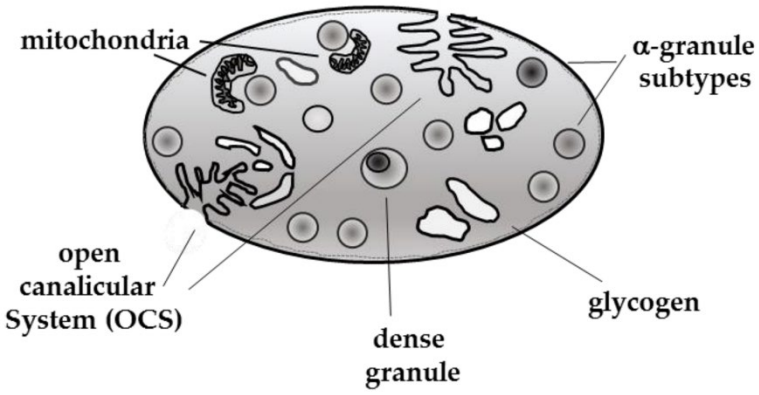


Figure 1 Schematic diagram of a canine platelet. Reproduced with permission from reference author: A.Pelagalli, University of Naples Federico II.[5]

The transmembrane and peripheral proteins act as receptors that allow signals for activation. The process from a circulating platelet to adhesion, activation and aggregation (primary hemostasis) is triggered when there is vessel damage enough to expose the endothelium through disruption of the glycocalyx. Activation can also be triggered in the intravascular space via a thrombin burst. Adenosine diphosphate (ADP) and soluble von Willebrand Factor (vWF) amongst other procoagulant factors are released from the damaged endothelial cells and collagen and vWF are exposed at the site of injury. Receptor signals are received on the circulating platelet surface and the platelet targets the site of injury. The platelet initially binds via the GP 1b-IX-V receptor to the collagen and immobilized vWF. This binding initiates the process of activation and shape change within the platelet. This includes

evagination of the canalicular system, which exposes further platelet receptors, most notably GPIIb-IIIa (CD 61/CD41), and allows release of the granular components of the platelet into the surrounding plasma space and triggers a profound platelet recruitment effect. The phospholipid bilayer also undergoes major alteration during platelet activation, including the translocation of the previously internally positioned negatively charged phospholipid phosphatidylserine (PS), that is a key component of thrombin generation regulation, to the outer layer. PS exposure on platelets results in assembly of intrinsic tenase (factors FVIIIa, IXa and X) and prothrombinase (factors Va,Xa and prothrombin) complexes, accelerating thrombin generation on the activated platelet surface.[6] Annexin V protein assay is one of the ways of assessing this key alteration stage, fluorescein marked annexin V binds to the externally positioned PS. Flow cytometry subsequently allows assessment of procoagulant activity in platelets and the loss of phospholipid asymmetry in cell membranes.[7]

There are three major membrane-bound granules within the platelet, alpha granules, dense granules and glycogen granules. Alpha granules contain coagulation factors (including fibrinogen and factor V), growth factors, glycoproteins, thrombospondin and fibronectin. The alpha granule membrane contains the alpha granule specific protein P-selectin as well as GPIb and GPIIb-GPIIIa.[8] Dense granules contain nucleotides and electrolytes (magnesium and calcium) and glycogen granules contain the energy source for the platelet. The process of release of the platelet organelle content is assisted by Lysosomes – that act to bring substances into the platelet by the process of endocytosis or act to fuse to the canalicular system to release proteins from the activated platelet. There has been recent work to identify the full nature of the proteomic release from the activated canine platelet (the platelet secretome).[9]

The activated platelet alters in shape subsequent to activation, with pseudopodia (filopodia) being formed that emanate from a small spherical centre. This allows the platelet to spread and adhere to the site of injury as well as aggregate, via fibrinogen bonds, to other activated platelets to produce the start of a clot.

As platelets progress through the complete process of activation, adhesion, aggregation and secretion the secondary phase of coagulation and clot formation occurs. This plugs the endothelial gap, which then further progresses to clot retraction via inhibitors and to eventual vessel healing.

There is a sophisticated feedback system in place that regulates the haemostasis process, to ensure that platelet activation and aggregation does not continue to occur once sufficient activity has taken place to allow an adequate primary response to the injury. This feedback has been investigated in the human model of primary haemostasis and includes downregulation of receptors and ongoing release of inhibitory substances such as prostaglandins and nitric oxide by intact endothelial cells.

Platelets not only have a role in primary haemostasis but the secretions from activated platelets are now known to play an active role in inflammation and wound healing. The proteomic content of the Canine Activated Platelet Secretome, having been identified in more detail, will allow the understanding of the pathophysiology of platelets in response to insult and disease to develop further.[9]

Thrombin generation and secondary haemostasis

Directly alongside the adhesion, activation and aggregation of platelets, secondary haemostasis is activated due to tissue factor (TF) expression/exposure in the subendothelium. When coagulation factors within the circulation (factor VIIa) bind to the tissue factor, the sequence of additional coagulation factor reactions that lead to the conversion of prothrombin to thrombin is triggered. Thrombin potentiates the change of bound fibrinogen to fibrin and thus begins the initial solid structure required to form a clot.[10] Thrombin itself acts as a potent platelet agonist initiating further platelet activity.[11] Thrombin generation is a measure that haemostasis is adequately activated to allow clot formation and is used as a quality control test for platelet products.

Pathologies involving platelets: Thrombocytopenia, thrombocytopathia and von Willebrand Disease

Thrombocytopenia

Thrombocytopenia is a quantitative lack of platelets within the circulation. The standard quantitative definition internationally is a platelet count of <100-200x10^9/l in both humans and canines. The absolute value varies in the literature dependent on the country and study interpreting the definition. Cause of thrombocytopenia can be broken down into four categories, lack of production, sequestration, destruction or excessive consumption

Lack of production: An issue at the level of the bone marrow. Examples are, neoplasia such as leukaemia, chemotherapy regimes, bone marrow (stem cell) transplantation and bone marrow aplasia.

Sequestration: Pooling of platelets, reducing platelet number in the remaining circulation. Examples are splenic torsions or haematoma, sepsis and neoplasia within vascular organs such as the spleen and liver.

Destruction: Considered to be immune-mediated and can be primary, due to antiplatelet antibody or upregulated T cell destruction of platelets, or secondary due to drugs, infections (viral, parasitic and bacterial) and neoplasia.

Excessive consumption: Injury, trauma or inflammation. Examples are severe haemorrhage, vasculitis and DIC.

The main focus of this literature review will be on severe thrombocytopenia, that would come into the category of consideration for platelet transfusions, which would equate to a platelet count of <50x10^9/l.

Thombocytopathia

Thrombocytopathia is a qualitative issue with platelets, leading to reduction in circulating platelet function.

Various thrombocytopathic conditions are recognized in both human and veterinary patients. They can be acquired in patients taking anti platelet drugs (such as aspirin or clopidogrel), or hereditary conditions.

In humans, examples are Bernard Soulier syndrome (macrothrombocytopenia and absence of GPIb-IX complex) and Glanzmanns thrombasthenia (lack of the adhesion platelet receptor GPIIb-IIIa).

In dogs, there has been specific breed associated platelet disorders recognized and reported in the literature. Pyrenean Mountain Dogs and a Golden Retriever have been recognized as having Glanzmanns thrombasthenia (GT), macroplatelets are recognized in the Cavalier King Charles Spaniel and the Akita.[12,13] Scott Syndrome, a lack of PS expression on the activated platelet has been documented in the German Shepherd and dense granule defects identified in American Cocker Spaniels.[5]

In situations of uncontrolled bleeding in thrombocytopathia patients, platelet transfusions can be part of their therapy, although current human approaches are directed towards initial use of alternative pharmaceutical or specific factor replacement before platelet transfusion where possible, for example factor VIIa in GT,[14] but platelet transfusions are still utilized and may be considered as a life-saving intervention.

Von Willebrand Disease

Von Willebrand Disease is a hereditary disorder that affects both human and veterinary patients, von Willebrand factor being crucial to the initial adhesion and activation of platelets rather than a platelet

disorder itself. In von Willebrand disease, there is a deficiency in this factor leading to inadequate primary haemostasis. Von Willebrand Disease is treated using replacement transfusions of concentrated blood clotting factors (cryoprecipitate) or recombinant von Willebrand Factor (VWF) not platelet transfusion.

Thrombocytopenia incidence in canines

In canines there is no large scale review available to indicate the incidence of thrombocytopenia in the general canine population. There are two retrospective case series from individual veterinary hospitals from 1991 in the USA and 2009 in Germany. These series looked at 987 and 871 thrombocytopenic canines respectively.[15,16] Thrombocytopenia was defined as a platelet count <200x 10^9/l or <150x10^9/l respectively. Overall incidence in the two hospitals was similar at 5.2% (987 cases out of 18910 cases seen over 6 years) and 6.7% (871 cases seen over 5 years) of total hospital patients respectively. Variance in the inclusion criteria and methods of analysis makes a direct comparison difficult but the epidemiology of the diagnoses showed some similarities as to the potential underlying pathologies. In the Grindem (USA) Paper, they looked at 987 cases between 1983-1989, 5% were Immune-Mediated Thrombocytopenia, 13% neoplasia, 23% inflammatory/infectious and 59% miscellaneous. In the Botsch (German) paper, they looked at 871 cases between 2000-2004. 5.6% were Immune-Mediated Thrombocytopenia (3.5% ITP, 2.1% Evans' syndrome), 28.0% neoplasia, 34.9% inflammatory/infectious and 25.5% miscellaneous. They also included a DIC category which represented 6%.

Immune-Mediated Thrombocytopenia and DIC were statistically associated with a lower platelet count than all other categories in the two canine papers. This suggests canine ITP is a condition that will be of interest when considering severe thrombocytopenia and its treatment in canines.

Across the broader veterinary literature, the most common diagnosis made in the presence of severe thrombocytopenia with requirement for potential platelet transfusion intervention in canines, is primary

Immune thrombocytopenia (ITP). [17,18,19,20] There are however many other pathologies in canines that have thrombocytopenia as a component. Advancing medical and critical care treatments for critically ill patients, advances in diagnostics, alongside more widely available veterinary oncology, chemotherapy and radiotherapy regimes, and surgical procedures such as those requiring cardiac bypass may well drive a requirement for more platelet product availability in this species.

Classification and diagnostic testing in canine thrombocytopenic disorders

Classification of thrombocytopenic disorders is not as well defined in canines as it is in humans. Internationally, for example, for human medicine, the World Health Organisation (WHO) produces a medical classification list of diagnostic coding termed the International Statistical Classification of Diseases and Related Health Problems (ICD) which illustrates the many specific diagnoses that can be made in people with thrombocytopenia.[21]

The Grindem and Botsch papers illustrate the limitations to definitive diagnoses in thrombocytopenic canines with between 25.5-59% reported as miscellaneous respectively. These limitations are also stated in many of the papers on investigation and treatment.[15,16,22–26] The standard approach to a thrombocytopenic canine is to perform diagnostic testing to exclude all known causes of thrombocytopenia, eliminate potential secondary triggers for ITP, and in that case, proceed to immunosuppressive therapy for the treatment of a suspected primary immune-mediated thrombocytopenia. Diagnostic testing to exclude other causes including secondary ITP would include full biochemistry, haematology and coagulation analysis, specific blood titres for parasitic disease, blood and urine cultures, imaging such as radiography, ultrasound and CT and in some cases bone marrow biopsy.

There is no highly sensitive or specific test available to definitively distinguish thrombocytopenia of non-immune cause, from ITP, or primary ITP from secondary ITP in dogs. The tests to identify anti-platelet

antibodies are considered to have low specificity and also, to achieve accurate results involve careful sample handling, as well as prompt testing (within 36 hours) making accessibility to practitioners challenging.[27]

Testing available includes direct and indirect methods, for IgG and IgM class antibodies, with the direct method identifying anti-platelet antibodies bound to the platelet surface and indirect methods detecting platelet-binding antibodies free within the serum. Testing modalities for direct antibody testing are immunofluorescence assay (IFA) and flow cytometry,[27] and for indirect antibody testing are radio-immune assay (RIA) and enzyme-linked immunosorbant assay (ELISA).

Thrombocytopenia incidence in humans

An epidemiological assessment of thrombocytopenia in humans to compare to the canine papers illustrated above is not readily available in the current literature. The focus in recent literature tends to be on specific disease entities. However, some countries have published thrombocytopenia incidence within individual institutions that allow some comparisons to be made. One Italian hospital reported cases with a specific ICD code for thrombocytopenia between 2004-2008.[28] This paper explored presented thrombocytopenia cases in more depth than the canine papers described. Their overall incidence of thrombocytopenia was calculated at 0.1% of their discharged patients, this equated to an estimated population incidence of 14.8 cases in 100,000 and nearly half of those were defined as immune-mediated thrombocytopenia (6.8 cases in 100000). Within the main study population between 2004 and 2008, 368 cases were identified, there was a 2.7% death rate, and a 7.6% incidence of clinical signs of bleeding, but none of the deaths were attributed to a bleeding complication. In this subset of patients, 65 out of the 368 cases (17.6%) required intervention, including splenectomy (12 patients), blood or platelet transfusion (31 patients), immunoglobulins (18 patients) or plasma exchange (4 patients). The same study group also analysed a further 40 patients with an updated

thrombocytopenia code in 2009, where 23/40 thrombocytopenic patients were specifically diagnosed with ITP. 57% of these patients exhibited minor bleeding, 7.5% major bleeding (WHO grade 2 or above) and one patient died of intracranial haemorrhage (ICH). 32/40 of these patients had a platelet count <30x109/l and 19 (47.5%) of these patients received a platelet concentrate transfusion.

In a further human paper looking at 245 prospective ITP cases in a UK geographical region, with a platelet count of <50 x 10^9/l between 1993 and 1999, there was a 1.6% fatality rate due to bleeding (4/245) and 30 (12%) presented with frank bleeding.[29]

Current human research papers are focused on thrombocytopenia within very specific categories, and normally those severe enough to require consideration for platelet transfusion. In humans, this would equate to a platelet level of <5-10x10^9/l, or if undergoing an invasive procedure 10-20x10^9/l, or <50x10^9/l with active recurrent bleeding as well as any additional risk factors such as drug therapy, ongoing inflammatory issues or disseminated intravascular coagulation (DIC).[30,31,32]

Immune thrombocytopenia in humans

There are two different immune led platelet bleeding disorders in humans worth consideration within this literature review, immune thrombocytopenia and thrombotic thrombocytopenic purpura (TTP). Immune thrombocytopenia (ITP), also sometimes known as immune-mediated thrombocytopenia (IMTP or IMT) and previously termed idiopathic thrombocytopenic purpura, until an international working group in 2009[33] kept the abbreviation ITP but defined it as Immune Thrombocytopenia (ITP). This nomenclature will be used for the remainder of this thesis. The quantitative definition of ITP was also defined as a platelet count of <100x10^9/l (so lower than the general definition of thrombocytopenia which is defined as a platelet count <150-200x10^9/l). They further recommended that the definition then be subcategorized to include primary ITP (autoimmune in origin, diagnosed in the absence of other causes or disorders that may be associated with the thrombocytopenia) and secondary ITP (all forms of

ITP except primary ITP) so for example, if the immune-mediated disease was considered associated with an infection such as *Helicobacter pylori*, the diagnosis would be "secondary ITP (*Helicobacter pylori*-associated)". Stages of disease were also defined, into newly diagnosed ITP (<3 months), persistent ITP (3-12 months) and chronic ITP (>12 months). Severe ITP describes an ITP patient with persistence of bleeding symptoms mandating immediate treatment, or the occurrence of new bleeding symptoms requiring additional therapeutic intervention above initial treatment. Refractory ITP is a separate category, to define ITP still persisting despite splenectomy.[33]

The pathogenesis of ITP involves the loss of tolerance to glycoproteins expressed on the surface of platelets and megakaryocytes.[34] The most commonly recognized IgG antibodies identified in 60% of ITP patients are anti- GPIIb IIIa and anti - GPIb/IX. There is also a separate category of antibodies, against phospholipids, that also bind to platelets, and antiphospholipid syndrome (APS) can also lead to thrombocytopenia.[35] A further area of interest relates to the presence of Human Leucocyte Antigen (HLA) antibodies in ITP patients. One study into 44 patients showed between 64-70% of ITP patients were positive for HLA class I antibodies.[36] Platelets express HLA on their surface. The potential role of HLA antibodies in ITP is recognized and is an area of ongoing interest. These antibodies are also considered to be contributory to platelet refractoriness. Platelet refractoriness is defined as two consecutive failures to respond to platelet transfusion, producing post transfusion platelet increments that are significantly lower than expected based on the number of platelets transfused and the patient's blood volume.[37] HLA matching is one of the methods used when patients become refractory.[30] Antibody coated platelets are considered to be removed by the spleen in a proportion of cases, hence the use of splenectomy in some ITP cases, but it has been noted that this is only effective in 2/3 of cases and it is considered that platelets are removed by the process of complement activation in others. GPIIb IIIa and GPIb/IX are also expressed on the surface of megakaryocytes so it is considered that there is a suppressive effect on platelet production in antibody-associated ITP. This does not explain the

proportion of patients with ITP who have no detectable circulating antiplatelet antibodies. Cell mediated immunity via the T cell subgroup of lymphocytes, and specifically cytotoxic T cells binding and lysing platelets, alongside a decreased number of T helper/regulatory cells is the proposed explanation for this subset of thrombocytopenic patients.[38]

Treatment of ITP in humans

Treatment for underlying disease or withdrawal of the offending medication, may well be all that is required for mild cases of secondary ITP. Treatment for primary ITP consists of the first line treatments of corticosteroids and Intra venous immunoglobulin (IVIG) that work via immunosuppression. Corticosteroids induce lymphocyte sequestration and destruction within the reticuloendothelial system and inhibit transcription factors that control synthesis of pro-inflammatory mediators, IVIG binds to T cells, B cells, FC receptors on macrophages and complement receptors,[39] inhibiting the production of more anti-platelet antibody by B cells and competing for mononuclear binding sites preventing platelet breakdown and lysis.

Anti-Rh D antibody can be used as a treatment for primary ITP in Rh positive patients with an intact spleen. The anti-Rh D binds to red cells causing the removal of antibody-bound red cells which reduces the removal of antibody-bound platelets. Splenectomy is used in primary ITP as a treatment option in cases that are considered refractory to, or show averse response to medical management, it is considered a viable treatment option in 2/3 of primary ITP cases where the spleen is the main organ involved in removal of antibody coated patelets.[34]

There is no definitive consensus but there have been studies into additional second line treatments for refractory ITP patients. Historically, the drugs considered are often further immunosuppressive therapy such as mycophenolate, vincristine and azathioprine.[40] More recently in human medicine, additional immunosuppressants have become third line treatments, subsequent to the development of two

categories of additional therapy, thrombopoietin (TPO) analogues that stimulate megakaryopoiesis and thrombopoiesis (romiplostim and eltrombopag),[41] and anti-CD 20 monoclonal antibodies (Rituximab). TPO analogues were the subject of a 2011 Cochrane review. They were concluded to significantly increase platelet number but there was uncertainty concerning their ability to reduce significant bleeding events and no conclusion could be drawn concerning overall survival. Rituximab is an anti-CD20 monoclonal antibody that binds and removes B cells associated with the production of anti-platelet antibodies. It is also thought that it may have additional immunomodulatory effect by normalizing T cell populations. There is however only one placebo controlled trial on this drug which did not show superiority when analysing endpoints in chronic ITP.[34,42] One further drug is currently within clinical trial and this trial is due to conclude in 2021. The TREATT (Trial to EvaluAte Tranexamic acid therapy in Thrombocytopenia) trial,[43] looks at use of Tranexamic acid (TXA), which is an anti-fibrinolytic agent. The trial is a double blinded randomized trial. Tranexamic acid is being evaluated not for its effect on platelet count, but on its proposed ability to reduce the clinical signs of bleeding by strengthening clot formation.

There has been controversy when considering the use of platelet transfusion in immune thrombocytopenia in human medicine. A longstanding consideration is that if a patients' own platelets are being destroyed by an antibody led immune response, additional platelets transfused may also have a shortened lifespan rendering them ineffective at their role in haemostasis or worse, adding to the patients morbidity due to the activated immune complexes and immune response initiated. However, in severe immune thrombocytopenia with critical bleeding, therapeutic platelet transfusions are used.[28,34,39,42] There is also a consideration that therapies such as Intravenous Immunoglobulin (IVIG) used alongside platelet transfusions may also help.[44]

Platelet transfusions in critical bleeding are given as required, and sometimes repeatedly - in one human study – one apheresis platelet unit was administered every 8 hours for a 72 hour treatment period with

concurrent IVIG, or as CRI's to try and control bleeding until the therapy for the condition starts to work. One study published platelet transfusion use in a high proportion of their particular subset of cases cases – 75% in a study looking at intracranial haemorrhage in children with ITP,[45] The overriding consideration for use in this instance being to control severe bleeding in cases that ongoing bleeding will lead to severe morbidity or mortality.[14]

Thrombotic Thrombocytopenic Purpura (TTP) is a separate immune directed condition in people that can be both inherited or acquired. It is associated with a severe deficiency in ADAMTS13 (a disintegrin and metalloproteinase TS13), which is critical to the cleavage of vWF. In acquired TTP, antibodies develop to ADAMTS13. This causes large platelet and VWF clumps to circulate, causing microthrombotic disease throughout the body, most commonly recognized in the renal circulation, but diagnosis is usually made when the following five symptoms are seen: fever, thrombocytopenia, microangiopathic hemolytic anaemia, neurological symptoms and renal insufficiency.[46] Its annual prevalence is thought to be 10 cases/million people. Mortality on initial presentation, with prompt initiation of plasma exchange (the mainstay of treatment) is considered to be 10-20%, and long term consequences are common. Platelet transfusion would be contraindicated in this condition.[14]

Canine immune thrombocytopenia

There are a few papers specifically on ITP in canines, and many others that explore it as an entity within other broader topics. There is some interest in the canine ITP model as a comparative model for human disease which is discussed later in this section.

Clinical ITP studies in dogs are predominantly based on retrospective incidence,[47,48] defining primary and secondary ITP, mortality rates and drug regimes to manage the condition.[19,22,42,49–53] Laboratory based studies focus on additional diagnostic testing in these cases to confirm immune cause (specifically antiplatelet antibody testing), but despite this work,[27,54–56] these tests remain out of reach of most

clinical veterinary trials. If antiplatelet antibody tests became more available, it would be interesting to see them within future clinical trials exploring thrombocytopenia diagnosis and treatment.[23]

Canine patients with naturally-occurring ITP usually present with symptoms of haemorrhage. Two studies reported an incidence of 70%[23] and 82%.[56] They often illustrate a high percentage of severe thrombocytopenia. 77% with a platelet count of <30 x 10^9/l,[23] bleeding is often surface (petechiae) but some present with more severe bleeding, ophthalmic, urinary, gastrointestinal and CNS. Melaena and an increased Blood Urea Nitrogen (BUN) are reported as a negative prognostic indicators.[51,56] ITP in canines has a published mortality rate between 3-48%.[47,53,55–59]

Primary ITP is reported as being more common in female dogs vs male (69%,65% and 73%).[23,47,48] It is considered there may be a breed related susceptibility for primary ITP, similar to other primary immune mediated diseases such as IMHA. Breeds considered to potentially have an increased incidence of ITP are the cocker spaniel, old English sheepdog, miniature and toy poodle and golden retriever, and further suggestion of potential familial risk in related dogs where more than one case was seen in related animals, but as ITP is a rare diagnosis, numbers for all of these studies are small.[15,55]

Investigation of a potential genetic causal link of immune mediated disease in dogs has been proposed and a specific study into cocker spaniels has been performed, looking at major histocompatibility antigens – specifically DLA (dog leucocyte antigen) but where a genetic link has been suggested alongside a heterogenous cause in people this has not been found in dogs at this time.[60,61]

Platelet transfusions are discussed as an option for severe ITP in dogs in many of the papers illustrating this condition – but currently have not been widely available or specifically studied in the canine patient[17]. Platelet dysfunction as well as depletion in platelet number may contribute to the severity of bleeding in dogs, suggesting that platelet number alone may not be the only factor contributing to the severity and symptoms a canine ITP patient may show.[54] A canine animal model of ITP where dogs were

injected with antiplatelet antibody to a target platelet count of 5-30x10^9/l also showed that bleeding occurred in all dogs – but that the extent of bleeding was variable and not dependent on platelet count alone.[62]

Immune thrombocytopenia and combined ITP and IMHA in dogs

ITP can be seen in combination with IMHA and when it is, is associated with a poor prognosis (76% mortality within 30 days of admission to a UK referral hospital).[25] Combined IMHA and ITP is also recognized in people and when diagnostic tests confirm a primary immune response to both the anaemia and the thrombocytopenia, it is termed Evans' syndrome.[63] It is rare that antibody tests are available to confirm the presence of antiplatelet antibody in the veterinary field, so although the combined syndrome is seen in dogs, it is not always possible to confirm the immune cause and hence the diagnosis of Evans' syndrome, but true Evans' syndrome may represent a proportion of these cases.[25] One study that did confirm ITP with antiplatelet antibodies alongside confirmed IMHA recognized 14 cases out of 106 cases of canine thrombocytopenia over a 6 year period.[23]

Canine experimentally induced model of immune thrombocytopenia

Canine/human comparative work in ITP can be found in the literature. A comparative medicine research group in North Carolina, developed an in vivo experimentally induced canine ITP model subsequent to initial work on a murine model. This work was published in 2014. The aim was to produce an animal model of ITP analogous to human ITP in order to investigate the disease further in terms of phenotypic presentation, and to look at the wider picture of immune modulation associated with the disease.[62] Within studies of thrombocytopenia in both humans and canines it is recognized that platelet count does not correlate with bleeding and it is hoped that this model will allow further research into the ITP disease process and indeed into potential future management and treatment strategies.

Within the North Carolina canine experimental ITP study, the authors looked at the experimentally induced ITP model alongside other naturally occurring thrombocytopenic presentations (6 primary ITP, one ITP secondary to ehrlichia and 4 thrombocytopenic dogs with other diseases). In both arms of the study it was recognized that platelet count in affected animals had no direct correlation to bleeding tendency as assessed using a modified bleeding score based on the human ITP bleeding score. This bleeding score (BLS) assessed 8 anatomical sites, providing a score for each patient out of a total of 16. This scoring system became the basis for the development of an ITP bleeding score for canines, the daily canine bleeding assessment tool, the DOGiBAT scoring system. The DOGiBAT system in comparison to the bleeding score (BLS) included the practical addition of bleeding from catheter/blood sampling sites to make up the full score, assessing 9 anatomical sites with a possible total score of 18.[64]

The experimentally induced ITP study showed similar baseline characteristics to naturally occurring thrombocytopenia cases used as a comparison within it, although all of the naturally occurring thrombocytopenia cases within their study had already received medications to treat their underlying condition (timeline not disclosed).

The experimental ITP arm of 5 dogs understandably was designed to not be life threatening, but to produce an in vivo model. Parameters in this group were measured at time 0 (considered induction of ITP) and 24 hours later, they received no treatment above ceasing the administration of the causal anti platelet (anti GPIIb) antibody. Over the 24 hour study period they showed an average decrease in Hct of 0.08 or 8% presumed due to their thrombocytopenic blood loss. All of these experimental induced ITP dogs survived.

Assessment of bleeding

Assessment of bleeding in both humans and canines is an area of ongoing interest and development. In dogs it is an area that there has not been consensus on in the veterinary field, previous to the development of DOGiBAT scoring in 2018.[64] Specific sites of bleeding were often used as descriptives in studies, or comparative human bleeding scores modified and utilized. DOGiBAT was developed so a scoring system could be used to assess bleeding similar to the generic WHO bleeding score (used in people with thrombocytopenia associated with malignancy and chemotherapy) and the human immune-mediated thrombocytopenia bleeding score (IBLS), but taking into account specific veterinary and canine considerations.[65] Scoring systems for thrombocytopenic patients have been constantly developed because there does not seem to be a simple correlation between platelet count, bleeding tendency and mortality in both people and dogs that can be used to reliably predict prognosis or guide therapy. Bleeding scales have been a focus of interest in the human field for 30 years. They have been found to be a helpful additional tool when looking at an entire clinical picture in order to continue to refine guidelines of treatment in bleeding cases, but it is still recognized that further improvements for recognizing more subtle sites of bleeding (such as small CNS or pulmonary haemorrhage) , as well as the extent of bleeding, may provide a better ability to guide on prognosis and therapy – but this may be outwith the reach of reality within many clinical trial settings.[66] These scoring systems are also challenged when assessing patients for trends in bleeding over a short timeframe (hours). When trying to use them to monitor improvements or deterioration, urinary, faecal and cutaneous bleeding cannot be relied on to illustrate an actual difference. Bleeding in these areas may reduce or increase, but faeces and urine may not be voided and even if they are, the clinical picture and testing available may lead to a record of exactly the same score, when in reality, a significant change may have occurred. This is illustrated by the removal of urinary and faecal bleeding assessments in one of the largest human platelet transfusion trials – the PLADO trial.[67]

Thrombocytopenia and the use of platelet transfusions in humans and canines

The use of platelet transfusion in thrombocytopenia in humans is well established.[30] The ability to produce platelet concentrate and transfuse became more common place around 1960, initially in leukaemia cases. Mortality due to bleeding in leukaemia patients was documented in hospital autopsy series to reduce from 63% (1963-1965) to 15% (1964-1965). This reduction was concluded to be due to the introduction of platelet transfusions.[68]

The use of platelet transfusions is routine in human medicine in many countries worldwide.[14,69] Platelet transfusions are part of the WHO essential medicines list.[70]

Although the role of platelets in immunity and inflammation is currently a topic of much interest,[71] their role in primary haemostasis in thrombocytopenia and thrombocytopathia is where they are currently utilized within medicine.

Current controversies in platelet transfusion

The current human debate involves trying to provide a robust evidence for how and when to administer platelet transfusions.[14] There is also a focus on whether there can be an overall reduction in transfusion events, due to the documented risks associated,[72] and also the recognized issues with platelet refractoriness in multiply transfused patients.[37] Both prophylactic and therapeutic strategies are used. This fine tuning of treatment in humans is due to well established protocols and the immediate availability of platelet transfusions, meaning multiple transfusions can be given as necessary, even to the extent of a constant rate infusion (CRI) to combat ongoing clinically significant symptoms. Due to this availability, mortality due to bleeding complications in thrombocytopenic humans in developed countries is low, but historically it was a more significant problem and is still documented. The cases most feared by human haematologists and indeed those most commonly associated with mortality or

severe debilitation in surviving patients, are those who develop intracranial haemorrhage associated with their disease.[34,45]

Platelet Transfusion – numbers of transfusions performed in humans

In the UK platelet transfusions are recorded to have occurred at a level of 220000 in 2007/2008 increasing to 275000 in 2014/2015 and dropping back to 250000 in 2017/2018.[14,73] The breakdown of these transfusions according to patient problem/diagnosis are 67% of all platelets are used in the management of patients with haematological malignancies, 7-10% in cardiac surgery, 5-9% in intensive care and the remainder are undefined.[14] The incidence of haematological malignancies is increasing, alongside the prevalence, due to increased survival rates. The mainstay of treatment of these disorders is myelosuppressive chemotherapy and haematopoietic stem cell transplantation, however this can lead to prolonged periods of severe thrombocytopenia. In 2010 over 50,000 stem cell transfusions were performed worldwide per year and there are many publications in this area.[74]

Platelet transfusion strategies and doses

Platelet transfusion strategies have been studied widely above the standard one "unit" dose which is described further in the platelet banking part of this literature review. This "unit" dose was historically developed by blood services based on calculations of the level of circulating platelets required to be infused in an average human patient in order to allow basic haemostasis to occur. For example in the UK one platelet concentrate unit is termed "one adult therapeutic dose" and the currently published goal of transfusion is to raise the platelet count by 20-40x10^9/l. This whole unit dose is proportionally reduced for paediatrics and further for neonates, based on bodyweight. Complexity comes in with this approach based on "expected rise in platelet count" due to the potential heterogenicity of the underlying cause of thrombocytopenia. Another way to approach platelet transfusion is to consider

the number of platelets transfused. A "unit" in the US contains 300–600×10^9 platelets which works out to be 50–100×10^9 platelets per 10 kg of recipient bodyweight. It is important to note when considering this, that platelet unit concentrate can have a variable number of platelets according to the donors platelet count and method of collection, but will conform to a minimum requirement via quality control testing. For example in the UK, NHS-BT test 1% of platelet units for platelet count, with both buffy coat pooled and apheresis platelets having to conform to a platelet count of >240x10^9 per unit in 95% of those tested.[75] The American Association of Blood Banks (AABB) and Council of Europe (CoE) have their own similar but slightly different regulations. In practice, even using set strategies, it is considered that the dose of platelets transfused can fall in the extremely wide range of 1·7–294·2×10^9 platelets per 10 kg.[76] Transfusion response will also vary dependent on the underlying condition and whether there is active ongoing loss, sequestration or destruction of both autologous (patient's own) and transfused platelets.

The overall aim is to provide an adequate dose of platelets to optimize patient outcome, while avoiding additional transfusions with their inherent risk and cost.[69]

Basic dosing strategy is explored in some of the earlier platelet strategy review work.[32] Reviews have been written,[69,74,77] guidelines developed and clinical trials have been performed that compare prophylactic vs therapeutic,[18] and low, medium and high dose strategies.[67,78] The overall recommendations from these studies are still not in full agreement, are specified for the different causes of thrombocytopenia in humans and indicate that further work is needed.[14]

A protocol of therapeutically transfusing patients where platelet count is <10 x 10^9/l alongside evidence of bleeding or where there is considered to be life-threatening bleeding (grade 4 on the WHO bleeding score) despite a platelet count >10 x10^9, using a standard platelet unit containing an average

of 300 x 10^9 platelets is generally accepted.[14] Prophylactic transfusion protocol recommendations from systematic review papers are less clear and more studies in this area have been suggested.[14,18,32,69,77]

Canine platelet transfusion indications

When we begin to look at clinical need for platelets in canines, and how best to investigate a product in thrombocytopenic cases, using human platelet transfusion studies, strategies and guidelines as a baseline is challenging. The simplest human study and indeed the highest use of platelets in humans is seen when platelets are decreased in malignancy and during and after stem cell transplants. Although thrombocytopenia is seen in canine malignancy,[16] chemotherapy regimes differ and stem cell transplant is currently unavailable in mainstream veterinary medicine, so a comparative use study in this area would be valid but hard to replicate.

Immune thrombocytopenia, from the small volume of peer reviewed literature available, appears to potentially be more common in dogs than it is in people, associated with more severe bleeding complications and with a much higher reported mortality rate (3-48%),[47,53,55–58,] than that reported in the human literature (0.1-5%).[29,63,79] ITP may also represent one of the most common presentations in dogs where platelet transfusion is considered.[26]

The approach to platelet transfusions suggested in the human field , of transfusion at a platelet level of <50x10^9/l with active recurrent bleeding has been suggested in the veterinary field, in the currently mainly theoretical situation of platelet use.[17,18,57] There are some recent studies involving platelet transfusion use, although the platelet trigger for therapeutic use appears slightly higher in the studies available (60 and 70 x 10^9/l).[19,20]

Platelet banking

Platelets are available through blood banking efforts, for humans worldwide. In the United Kingdom (UK) alone the human National Health Service Blood and Transplant (NHS BT), biennial report 2017-19 reported provision of over 250,000 units of platelets per year.[73] Across Europe 2.4million units, and in the US, approximately 1.3 million units of platelet concentrate were transfused in 2015.[80] Population growth, an ageing population and advances in oncological and cardiothoracic surgery appear to be driving this number upwards.[73] Platelets are used in humans for both prophylactic (at risk of bleeding due to thrombocytopenia) use, or therapeutic (quantitative and symptomatic thrombocytopenia) use, most commonly for the treatment of neoplasia. High levels of platelet transfusions are administered to people receiving chemotherapy during preparation for Bone Marrow Transplant. Platelets are also used for a wide range of other problems.[30] Platelet transfusion provision is regarded as an essential medicine by the World Health Organisation (WHO).[70]

There are challenges involved with collecting, processing, storing and using platelets for transfusion therapies. Fresh platelets have a short shelf life of 5 days, require constant agitation and storage within a narrow range of temperature. In human medicine, platelet use is associated with the highest risk of transfusion transmitted infection (TTI) of any blood product used for transfusion.[81]

In order to contain a therapeutic level of platelets, i.e. an adequate concentration/actual number of transfused platelets to allow primary haemostasis to function as an effective process in a thrombocytopenic patient, a standard unit of platelets for transfusion to one individual patient is derived from 5 standard 450ml whole blood donations (i.e. from 2.25litres of whole blood), or collected by the process of extracorporeal blood filtration: plateletpheresis (apheresis).[82]

Apheresis is more expensive due to the specialist machine needed, consumables required, and takes longer for a donation to take place than a standard collection of whole blood. However, in human

studies, apheresis platelet concentrate (APC) when compared to whole blood derived platelet concentrates, from either platelet rich plasma (PRP) or from buffy coat (BC), via meta-analysis, shows a significantly higher corrected platelet count increment at both 1 hour and 18-24 hour post transfusion.[83] APC may also be considered beneficial due to the platelets being derived from only one donor, with one blood type, one set of disease risk and a reduced incidence of potential bacterial contamination events (skin plugs at venipuncture).[81,82]

Canine platelet source and availability

In the United States (US) there are commercial (profit and not for profit) canine donation programmes, collecting, in 2011, an estimated 20,000 units of canine whole blood per year. Most units collected are processed into the common components, i.e. packed red cells and fresh frozen plasma. Some are also distributed as whole blood, and a small proportion of units are processed to produce platelet products. These units are sold to the veterinary profession. In contrast, the US human blood bank services collect approximately 16 million units of red cells each year.[84]

There are a small number of donation programmes within some veterinary hospitals and some licenced colony programmes, carrying out plateletpheresis.

There is a range of platelet transfusion products, with variable availability, which can be accessed by the veterinary industry in the US. The current transfusion options for dogs requiring platelets include:

- Fresh platelets “random donor platelets”: canine platelet rich plasma (PRP) or canine buffy coat (BC) derived platelets from one 450ml unit of whole blood.
- Fresh platelet concentrate: pooled PRP/BC platelet concentrate, standardly derived from 5 units of whole blood. Fresh platelet options are usually collected and supplied by on site blood banks and their production and supply is extremely limited.

- Frozen Platelet concentrate: dimethyl sulfoxide (DMSO) stabilized product with a frozen shelf life of six months
- Lyophilized platelets: StablePlate RX®, a trehalose stabilized lyophilized platelet concentrate with a current 2 year shelf life at room temperature.
- Fresh Apheresis derived platelet concentrate. This has very limited availability in the US. The difficulty and high cost (currently $1600 – North West Veterinary Blood Bank - for a single unit containing $2.5x10^{11}$ platelets with a 5 day shelf life at room temperature) of providing a constant and timely supply of fresh apheresed platelet concentrate to veterinary centres across the US has been documented.[20]

In the United Kingdom (UK) there is one canine charity blood bank (Pet Blood Bank UK (PBBuk)), which supplies canine blood products to veterinary surgeries around the UK. This operates under Veterinary Medicines Directorate (VMD) licence and collects around 2500 whole blood units a year (2016). There is one commercial blood bank that supplies its own clinics - Medivet Vet Blood - that operates under VMD licence. Alongside these two licenced blood banks, there are multiple referral centres that run their own non- licenced programmes which by their nature can only supply transfusion products to their own registered patients under Royal College of Veterinary Surgeons (RCVS) guidelines.

Neither of the two licenced UK blood banks currently offer any platelet product option. There is one European Blood Bank (Banco de Sangue Animal) that imports products into the UK under an animal byproducts certificate (2017), that provides frozen platelet concentrate sourced from volunteer canine donors in Portugal.

Canine fresh PRP is very rarely available in the UK, if at all (it may be being made in specific referral institutions on a patient need basis). Hence within the UK, there are rarely any platelet transfusion options for the majority of dogs that are suffering from bleeding due to thrombocytopenia as there is in

the US, or in humans. Fresh whole blood transfusion is usually the only option other than pharmacological or surgical treatment for the underlying problem. A standard unit of 450ml whole blood contains on average $5.5x10^{10}$ platelets. A standard unit of platelet concentrate in humans to therapeutically manage thrombocytopenia pooled from multiple units of whole blood or by apheresis, contains $3\text{-}6x10^{11}$ platelets per unit. Therefore platelets from at least 5 units of whole blood are pooled to produce one therapeutic platelet concentrate unit.

In a study investigating the efficacy of low ($1.5\text{-}3x\ 10^{11}$) vs standard ($3\text{-}6x10^{11}$) platelet transfusion doses in thrombocytopenic human patients (Strategies for Transfusion of Platelets - SToP randomized controlled trial 2009), the trial was stopped before completion due to significant bleeding in the low dose arm during an interim analysis.[31,78]

Fresh Whole Blood transfused within hours of collection at room temperature is often discussed as an available option for veterinary patients needing platelets. In a large canine patient (40kg or more) with a similar level of thrombocytopenia to human transfused patients, this would equate to multiple units of fresh whole blood being required (Table 1). Fresh whole blood transfusion was rejected in the 1960's in human medicine. The recognition that pooling platelets into concentrate form allowed an appropriate transfusion dose to be administered without the risk of volume overload was the main consideration to support this. An additional benefit was reduction in the risk of red cell sensitization. Component therapy also allows red cells and plasma to be used in other patients.[85]

Conditions causing thrombocytopenia in the canine are less well defined than in humans and are potentially more severe when recognized. Further research is required to address knowledge gaps in the role of platelet therapies in the management of canine thrombocytopenia.[17]

Canine Weight	Canine BSA m^2 [86]	1.1x 10^{11} platelets per m^2 – dose and volume of fresh whole blood required in mls	2.2x10^{11} platelets per m^2 – dose and volume of fresh whole blood required in mls	4.4 x 10^{11} platelets per m^2 – dose and volume of fresh whole blood required in mls
5kg	0.3	3 x 10^{10} – 245ml	7x10^{10} -573ml	12x 10^{10}-981ml
20kg	0.74	8x 10^{10} – 656ml	16x10^{10}-1311ml	32x10^{10} -2600ml
40kg	1.18	13x10^{10} – 1066ml	26x10^{10}-2131ml	52x10^{10} -4262ml

Table 1: Fresh whole blood (FWB) doses for thrombocytopenia. Illustrating an extrapolated platelet dose based on the three dosing strategies used in the determination of the optimal prophylactic platelet dose strategy to prevent bleeding in thrombocytopenic patients (PLADO) trial for severely thrombocytopenic people.[67] Doses of 1.1x10^{11},2.2x10^{11} and 4.4x10^{11} platelets were used per m2 body surface area (BSA). This table compares these doses for commonly encountered weight categories for canine patients and illustrates the volumes require to meet these doses when using fresh whole blood. For the purpose of calculating BSA in dogs, the British Small Animal Veterinary Association (BSAVA) conversion table was used.[86]
For the purpose of this table – one canine fresh whole blood unit - 450ml volume is considered to contain 5.5x10^{10} platelets or 0.0122x 10^{10} platelets per ml

Fresh whole blood transfusion involves the administration of red cells and plasma as well as platelets which, as demonstrated in table 1 represents a significant transfusion volume when administering a quantitatively appropriate (i.e. adequate number of platelets) dose. These volumes and the potential known reactions associated with red cell and plasma transfusion from multiple donors, pose risk to the patient that may outweigh benefit.[18]

Current UK veterinary blood banking programmes, their legislation and size make collection of large numbers of canine blood donations challenging. Large numbers of donations are needed to produce platelet products. Consequently, it is unlikely in the near future that a UK sourced platelet product will become readily available to UK veterinary practitioners.

Is there an unmet need for more platelets?

Provision of platelets or their alternatives and their need in human transfusion medicine is well established, extremely important, and life- saving. For over fifty years there has been an effort within the blood banking scientific community to conceive and generate a platelet product that is a solution to assisting primary hemostasis in areas where availability of standard transfusion of platelet products may be logistically challenging.[87]

In the human field, the use of platelets for haematological need in oncology patients can be met with the current systems so long as donation programmes remain robust. Use of platelets for cardiothoracic surgery, such as bypass, can be planned and catered for by current national blood bank supply chains. These are the NHS BT (UK) and the U.S Food and Drug Administration (FDA) regulated blood banks such as (but not limited to) the Red Cross (US). There is no great driving force for change. However, as research continues, it is considered that demand may increase. Scenarios such as catastrophic radiation toxicity, when modelled, highlight a potential need for rapid access to large volumes of primary haemostatic support.[88]

The risks associated with conventional platelet products such as disease transmission and bacterial contamination should also be considered.[81] If efficacy can be maintained , the risk of transfusion transmitted infections may theoretically be reduced by transfusing a platelet product that has been through additional processing steps, involving heat and freezing during their manufacture.

Currently, a large force for change comes when we consider the situations outwith developed blood banking systems and standard hospital pre planned care, where geography, demographics, ambient temperature, storage facility and shelf life result in conventional platelet product transfusion becoming practically challenging or indeed impossible.

Platelet consumption in massive trauma cases and intensive care is an area where there is much current interest and research within both human and animal emergency and critical care facilities. Platelet transfusion may well become a consideration in the initial stabilization of this subset of patients.[89]

Prompt use of platelet products to enhance primary haemostasis, at times of compromise or overwhelming demand, may potentiate conventional transfusion therapy, reducing both subsequent transfusion need as well as mortality.[89]

Considering this scenario, acute trauma medicine providers where products require to be carried in the back pack of military personnel, or first responder emergency services such as air and land ambulances would benefit from a practical and efficacious solution to their platelet transfusion requirements.[90]

A smaller but aligned parallel can be drawn for the global veterinary industry, where treatment regimes tend to follow human trends, but are composed of privately funded, low case load, geographically isolated, hospital based systems that provide advanced medical care. This is not an area that can be easily or realistically supplied even by the largest of veterinary multispecies blood bank, if we consider transfusion of fresh platelets or frozen platelet concentrates as the only options.

Lyophilized platelets are not the only potential solution – but they may provide what appears to be one practical answer to these logistically challenging scenarios.[80] Lyophilized platelets are presented as a freeze dried pellet, within a sterilized vial, reconstituted with sterile water for injection and given as a slow intravenous bolus injection. With a shelf life of at least 2 years with storage temperatures between 4-30 ^{O}C, these products may be a much more versatile and robust option than conventional platelet products. As a pooled product they have a verified number of platelets per millilitre of reconstituted solution, allowing individual calculation of dose according to patient size and the underlying condition to be treated.

Regulation and quality control of platelet products for transfusion

All human platelet products within the licensed blood banking community go through a series of quality control testing prior to transfusion to determine standardized measures of safety and efficacy.

Across the world, the regulation of blood banks is paramount for the production of safe and efficacious transfusion solutions. This is advocated by the WHO (World Health Organisation).[91] In the UK, US and across Europe there are many well established organisations that define and regulate strict protocol and manufacturing principles that need to be followed, according to the countries individual government licensure. The National Health Blood and Transplant Service in the UK is the sole supplier of Human Blood for transfusion and is regulated by the UK government Department for Health and Social Care. In the US the FDA legislate and regulate, and the American Association of Blood Banks (AABB) accredit many blood banks to supply the US market. These are examples of some of the largest organisations that coordinate human transfusions from collection to product supply.

In the Veterinary blood banking world this structure is less established, with only the UK requiring a Veterinary Medicines Directorate licence to operate a non-food animal blood bank (NFABB). Most international veterinary blood banks follow comparative human guidelines (from their licenced human counterparts) but have very variable degrees of legislation and regulation.

Blood products in general carry a specific categorization that usually does not come under the standard of a “drug” and therefore they often do not carry specific drug product licences.

A key part of regulated transfusion medicine is that products can be followed and individually identified at all times from donor through to recipient. Consistent quality control systems and robust haemovigilance procedures would be considered an absolute requirement.[91] In this constantly

developing area, ongoing research and development leads to ever expanding knowledge. Through this, blood product safety and efficacy can be constantly improved.

Lyophilized platelets

Lyophilized (freeze dried) platelets have been under development for over fifty years.[87] Initial stabilization efforts were hampered by loss of therapeutic efficacy when tested in vitro or in vivo in bleeding models.

The process of freeze drying a cell to preserve its integrity and biological function is exceptionally complex. A key requirement is to stabilize the cell initially using a method of cross linking before trying to dehydrate and freeze it. This was initially found to be effective using a paraformaldehyde preservative solution prior to freeze drying, in 1975. However, despite preservation of structural platelets to rehydration as visualized by electron microscopy, with the additional observed ability to agglutinate in the presence of vWF, these initial lyophilized platelets on further testing were found not to be biologically effective at stopping bleeding in the clinical model.

Modification of the paraformaldehyde preservation was continued by a Carolina based working group to produce a clinically effective product on transfusion into a thrombocytopenic rabbit model, in a surgical cardiac bypass model in dogs,[92] and in a porcine haemorrhagic shock model (in combination with a haemoglobin Oxygen Carrier (HBOC)) and reported between 1994-2008.[87]

Trehalose preservation is an alternative method of preserving platelets that has been investigated and refined since 2001.[93]

Preclinical testing has shown efficacy in the bleeding thrombocytopenic rabbit model, severe haemorrhage due to liver lobectomy in non-human primates (NHP) and canine cardiac bypass models.[94] A summary of some of the data and conclusions from initial safety, dose escalation, comparison to

current human treatment standard (fresh platelets) and efficacy trials including canine coronary bypass can be found in Appendix 1.

Development is continuing and due to the canine model used in the pre-clinical,[92,94,95] and subsequent clinical trial work, the canine version trehalose buffered freeze dried platelets reached the US veterinary market in September 2018 as Stableplate Rx® and this is in routine use across the US in canine thrombocytopenia cases. Prototypes have been manufactured and trial work continues into rabbit, equine and elephant derived lyophilized platelet products.

The human product, Thrombosomes®, has progressed through stage one clinical trials in human patients and is now progressing into FDA approved stage 2 clinical trials in thrombocytopenic oncology patients in the US, as of April 2020.

Lyophilized platelet manufacture, quality control and preclinical testing

Canine lyophilized platelets are produced within a separate veterinary clean room that mirrors the GLP and GMP compliant laboratory facilities that manufacture the human product Thrombosome®. This is a unique model and there is no similar comparative programme for veterinary transfusion solutions anywhere else in the world. The two entities of human and veterinary blood banking internationally may have many similar protocols, but do not mirror each other in standards and regulation.

Platelet concentrate is sourced on the canine side by apheresis from closed colony hounds and beagles kept in USDA licensed AAALAC (Assessment and Accreditation of Laboratory Animal care) approved sites. All donors are blood typed for Dog Erythrocyte Antigen (DEA) 1 and 7. All dogs are tested negative for anti-DEA plasma antibody to all known DEA types. These donors receive preventative care as a standard, full vaccination to canine distemper, parvovirus, infectious canine hepatitis, parainfluenza, *Bordetella bronchiseptica*, leptospirosis and rabies. They are tested quarterly for

infectious diseases according to the consensus statement from the American College of Veterinary Internal medicine on testing for blood-borne pathogens in canine and feline blood donors.[96]

Once apheresis derived platelet concentrate is obtained in a batch (12-20 units), the quality control testing before and after lyophilization, lyophilization procedure and final product control is the same for both the human and canine product. The laboratory operates under GLP and GMP guidelines and is regulated by the FDA.

Pre manufacturing quality control involves an assessment of "swirl" (gentle agitation to visualize discoid platelets that reflect light. Swirling is seen as an indication that platelets are not activated and are expected to be functional in vivo at the time of transfusion), lactate, particle size and platelet count.

Post lyophilization batches are tested for:

- Bacterial Endotoxin test (BET) using limulus amebocyte lysate (LAL)
- Anaerobic and aerobic culture to identify any bacterial contamination
- Particle size to ensure that there is not an unacceptable level of particles <1.03µm which would be defined as a microparticle or >5.04µm which would indicate aggregation, 1.03-5.04µm is the accepted size for a platelet.
- Identification of Platelet Surface Markers involved in platelet activation and aggregation. GP1b, GpIIb-IIIa (CD 41-61), and annexin V
- Measurement of adequate aggregation and clot formation, via thrombin generation assay

Lyophilized platelets, preclinical assessment of circulation kinetics

Circulation kinetics of lyophilized platelets have been studied in the New Zealand white rabbit (NZWR) – using both Thrombosomes™ (human derived lyophilized platelets) and rabbit lyophilized platelets. Clearance is diphasic with a significant drop in circulating lyophilized platelets within 10 minutes and

majority clearance (60-70%) within 2 hours.[94] The remaining 30-40% demonstrated a half life of around 24 hrs .[97]

This is more rapid than the clearance seen with fresh platelets.

Areas of ongoing interest in the field of platelet transfusions where lyophilized platelets may have relevance.

Antigenicity of platelet products

The antigenicity of lyophilized platelets is an area of interest. A recognized complication in patients who have to receive multiple platelet transfusions over time, is refractoriness to further transfusions due to the development of anti-platelet antibodies.[37] Lyophilized platelets have been compared to standard refrigerated platelets. In previously sensitized NZWR receiving standard refrigerated platelets, alloantibodies do develop and a rapid clearance of refrigerated platelets is seen on subsequent exposure. This has not been recognized in rabbits that have previously received lyophilized products that are multiply transfused. As antigens capable of causing alloimmunization exists on lyophilized platelets, shown in testing post lyophilization, the reasons behind this are not clear, and much more research is needed. However, this phenomenon may be associated with the rapid action and equal rapid clearance of these partially activated platelets.[94]

Platelet Refractoriness

Platelet refractoriness is an area of interest in the human transfusion community as it can represent a significant clinical problem.[98] Human patients can require multiple platelet transfusions throughout their lives for hereditary disorders, or throughout the timespan of an illness such as a haematological malignancy. It is preferable that any transfused platelets are not removed prematurely as that may necessitate an increased number of transfusion events and subsequent increase in risk to the recipient.

Incidence of platelet refractoriness in France in 2015 was reported in 12.7/100,000 platelet concentrate transfusions.[30] Platelet refractoriness is considered to be 80% caused by non-immune causes, such as sepsis, DIC, ongoing bleeding and splenomegaly.[37] Immune-mediated platelet refractoriness is predominantly considered to be caused by Human leucocyte antigen (HLA) antibodies. Leukoreduction of platelet transfusion products is a step that has reduced HLA exposure and subsequently reduced refractoriness, as although HLA is expressed on platelets themselves it is thought that transfusion of white cells within platelet products potentiates the development of antibodies further. Other Human Platelet antigen (HPA) antibodies are also implicated. Ultraviolet B light treatment has been introduced in some programs and has been shown to reduce alloimmunization and refractoriness.[83]

Whether using whole blood derived pooled platelets from multiple "random" donors or apheresis platelets from a single donor, most human transfusion services now ABO and Rhesus (Rh) type platelet donors and issue ABO and Rh-type matched platelets. This is to prevent alloimmunization to non self A or B antigen that may be present on residual red cells in the platelet product, but are also expressed on the platelet surface (in low levels). Type matching also prevents low levels of haemolytic red cell removal in the recipient due to anti A, B or anti Rh antibodies in the residual plasma.[99] To reduce blood type associated reaction, emergency use (i.e. in a recipient of unknown blood type) in the US utilizes, O Rh +ve platelets , in the UK, A Rh +ve platelets. The discrepancy is due to a divided consensus as to the most significant concerns between the regulatory bodies who devise the protocol in the US and the UK. Thirty to seventy percent of multiply transfused thrombocytopenic patients become refractory to random-donor platelets. ABO type matching for platelet transfusions is considered to be relevant due to the points mentioned above, but some studies have suggested that it does not alter overall clinical outcome in patients.[99,100] Platelet cross matching,[37] or recipient testing for alloantibodies (to HLA or Human Platelet Antigens – HPA) can be performed and matched platelets can be sourced to reduce the incidence of refractoriness to platelet transfusion.[101]

Platelet refractoriness in the canine

A study into platelet refractoriness used a dog model as part of a large human trial looking into reducing alloimmunization using leukoreduction and UVB light treatment. The method was to administer canine platelet transfusions manufactured from small volumes of whole blood and administer them frequently to recipient dogs over a period of 8 weeks. This study clearly illustrated that dogs do develop alloimmunization, and do become refractory to platelet transfusions. Although this has not been published within a clinical canine case series, we therefore know that like in humans if multiple platelet transfusions are given to clinical cases, it is likely that we will see the same alloimmunization and refractoriness in dogs that we see in humans.[102]

Pathogen reduction

An area that is not fully explored with lyophilized platelet products is their potential superiority in preventing transfusion transmitted Infection. Despite rigorous testing protocols in place for donors and resultant transfusion products – with emerging viruses including retroviruses – pathogen reduction and inactivation are areas of current interest in the transfusion community.[103]

This has been mentioned in early lyophilized product overviews,[87] with postulation that the stabilization and freeze drying process would deactivate many known pathogens. It has been suggested that a sterile product may potentially be achieved.

Adverse events (AE) to platelet transfusion

Adverse events (AE) or reactions to platelet transfusions are recognized,[80] and it is not possible to fully mitigate against reaction when using any transfusion product. Platelet products are associated with the highest level of reaction across all transfusion products.[72] There have been steps made to reduce these reactions. Platelet donor selection now involves not using female donors who have been

alloimmunised by pregnancy. Platelet transfusions are ABO type matched for donor and recipient. There has also been steps made during collection and processing that include skin plug diversion, leukoreduction and minimizing residual plasma by replacing it with platelet additive solution (PAS). Adverse events associated with platelet transfusion are currently mainly confined to febrile, allergic and hypotensive reactions in humans[72,104] In France, in 2015, adverse events (reactions) to platelet concentrate transfusions were reported as 441/100,000[30] and in the UK in 2016 38.9/100,000.[104] The tenfold difference in these levels may be due to different criteria within the haemovigilance reporting structures in the two countries. Milder reactions may make it to the French report, whereas they may be discounted by Serious Hazard of Transfusion (SHOT) UK report. There were also differences in the timing between the UK and elsewhere concerning quality control protocol such as bacterial screening, skin plug diversion methods and the use of platelet additive solutions (PAS) for platelets. These came into place in the UK system prior to being implemented elsewhere (personal communication with NHS BT). The most concerning and serious reaction is that of bacterial contamination of platelet units and potential sepsis in recipients. In France in 2015, there were 2.9/100,000 severe or fatal reactions due to bacterially contaminated platelet transfusions reported.[30] In the UK 33/40 transfusion transmitted infections (TTI's) reported via the SHOT haemovigilance scheme from the start of reporting were ascribed to Platelet transfusion and were associated with 28% mortality. Since the introduction of bacterial screening of platelet units in the UK in 2010, there have been no additional TTI's reported, but any severe febrile reaction is still investigated as a TTI as it is recognized that false negatives in screening can occur.[14]

Canine clinical trials

Summary of platelet transfusion clinical trials in canines

Relevant clinical trials include a trial that explored the use of DMSO cryopreserved platelets in 43 thrombocytopenic dogs over a six year period (2007-2013) in one institute, comparing them to a further 43 thrombocytopenic dogs who did not receive platelets. This study reported 56 (65%) ITP across the two groups and of these 56, eight were noted to have combined signs of IMHA. Interestingly 60% of the enrolled patients receiving one platelet transfusion as part of their treatment required further blood products as did 28% of the control arm. Transfusions administered included red cell and plasma. As this was a retrospective study, the more severely affected presentations tended to make it into the treatment arm, so true comparisons and conclusions are hard to draw between the two groups, but the authors concluded that there was no significant difference in length of hospitalization, survival to discharge or mortality between the groups.[19]

A prospective study looked at formaldehyde stabilized lyophilized platelets vs fresh canine platelets in 37 dogs presenting at 5 referral hospitals in the US over a 9 month period (2008-2009). This study included 27/37 (73%) with primary ITP and an additional 4 (11%) with combined ITP and IMHA. All dogs enrolled in this study had a bleeding score (BLS) >1, so some signs of haemorrhage and 47-50% depending on the treatment arm required additional blood transfusion support beyond the platelet product administered as part of the study. There was no significant difference found in efficacy when considering platelet count, haematocrit, bleeding score or mortality between fresh platelets and lyophilized platelets.[20]

A further prospective study that published in 2020 illustrates the administration of cryopreserved platelets vs trehalose buffered lyophilized platelets, enrolling 88 bleeding thrombocytopenic dogs across

12 centres. Fifty dogs received Lyophilized platelets and 38 received DMSO cryopreserved platelets. The study did not define diagnoses in the 88 enrolments,but stated that the study population was heterogenous consisting predominantly of cases of ITP. This study reported a much lower additional transfusion percentage of 13/88 (15%), consisting of additional platelet product (3) and packed red cells (2) within the 24 hours study period and eight further transfusions during hospitalisation, including packed cells (4), fresh whole blood(2), cryoprecipitate (1) and albumin(1). This study suggested that lyophilized platelets are not inferior to DMSO cryopreserved platelets and in the acute timeframe (1 hour post transfusion) may be superior to DMSO cryopreserved platelets for reduction in bleeding score and limiting a decrease in haematocrit.[26]

All of these studies indicate that the most frequent diagnosis associated with the requirement for platelet transfusions in canines was ITP

Platelet transfusion in canines

A state of the art review in 2009 explored the platelet transfusion options in canines and concluded that platelet transfusions may be essential to the management of dogs with severe thrombocytopenia or thrombocytopathia experiencing uncontrolled or life-threatening bleeding.[17] Concerning Lyophilized platelets, the main conclusion drawn in the reviews and studies involving them, is that because of the short life span of lyophilized platelets they may be best used as a haemostatic agent in the face of catastrophic or life-threatening bleeding while initiating more definitive treatment. Their practical advantage of long shelf life, ease of storage, fast reconstitution and administration may allow them to be a rapidly accessible bridge in a patient with life-threatening haemorrhage.[17,20]

Clinical trial approach and design

In human medicine, the approach to clinical trials when looking at a novel treatment is a progression through preclinical safety and efficacy testing, to smaller clinical trial safety and efficacy in the patient and eventually onto larger scale clinical trials. Clinical trials looking at safety and efficacy of an intervention, wherever possible, are currently designed in a prospective randomised control trial (RCT) format. The RCT format aims to provide non-biased evidence about the intervention being investigated. Randomized clinical trials still have their drawbacks, the most obvious the level of time and investment usually required to run them.

There is also still within RCT format, possibilities for poor design, challenges with heterogenous populations, difficulties enrolling adequate numbers, unclear endpoints, missing data and bias from study authors when interpreting data.

Examples of some prospective RCT within the field of human platelet transfusion and thrombocytopenia are the StOP trial,[78] which looked at standard and low dose platelet transfusion strategy, the PLADO trial,[67] which looked at three levels of dosing for prophylactic platelet transfusion and ongoing, and an example of a blinded trial looking at one particular treatment, the TREATT trial,[43] looking at tranexamic acid treatment in thrombocytopenia.

UK legislation meant that although safety had been explored in pre-clinical work and in two US veterinary clinical trials, safety analysis would need to be part of any UK study. Uniquely in the UK in comparison to the US, due to the absence of any currently available platelet containing product, efficacy could be assessed by comparing patients receiving no platelet product at all vs lyophilized platelets. Both previous US studies involving lyophilized platelets have compared lyophilized platelets to a different platelet containing product, as that would have been considered standard of care in the US. For the first time, in this UK study, it was also possible to design a trial that could be blinded, because of the ability to manufacture a control solution that was indistinguishable from the test solution to the

clinician using it. These considerations allowed the UK study to be designed looking at a unique question and as an RCT that followed on from conclusions within the literature available.

Within veterinary research, it is recognized that randomized controlled trials (RCT) are uncommon and can be challenging to perform for many reasons.[105] However it was felt that with a product of this nature, although challenging, a RCT was the right trial design to use.

While designing the trial, the initial research question was to identify appropriate clinical trial endpoints when looking at a heterogenous condition, alongside defining an achievable and realistic enrolment number and criteria. Additional areas of focus were to define how to analyse the results, critically evaluate the trial and the results, and present conclusions in relation to lyophilized platelet use in canines, including the recognition of potential future research direction. At the conclusion of trial design, the aims, objectives and hypothesis for the trial were set.

The aim of the study was to assess the safety and efficacy of trehalose buffered lyophilized platelets in severely thrombocytopenic canine patients.

The objective of the study was to compare the test group receiving lyophilized platelets to the control group receiving control solution. The safety objective compared adverse events between groups over the 24-hour study period. The efficacy objective compared changes in bleeding score (DOGiBAT), Haematocrit (HCT) and Platelet count (PLT) at three time points within twenty four hours of administration of trial solution, between groups and across timepoints.

The hypothesis was defined that trehalose buffered lyophilized platelet transfusion, when compared to medical management alone, would significantly improve key clinical endpoints related to bleeding due to thrombocytopenia, and that significant adverse events would not be demonstrated associated with their administration.

Clinical Trial

Introduction

When severe thrombocytopenic cases with signs of active bleeding present in canines, it is often a critical presentation with a high morbidity and mortality. The most common aetiology is immune thrombocytopenia (ITP).[19,20]

Current approach to the treatment of thrombocytopenia in canines in the UK is to treat the underlying disease alongside using available transfusion support such as fresh whole blood or packed red cells in cases that are also anaemic. In ITP first line treatment is focused on immunosuppression. Treatment response can be unpredictable, as recovery of adequate circulating platelet number in order to allow haemostasis can take time. While severe thrombocytopenia persists, the risk of critical bleeding remains present.

Platelet concentrate transfusion is the standard of care treatment in human medicine in the developed world for severe thrombocytopenia with risk of, or apparent active bleeding. This is a treatment option recognized by the WHO as essential.

Platelet transfusions are not available to the majority of veterinary practitioners to treat their canine patients with thrombocytopenia. There is a consideration that they may be of benefit, especially for the management of uncontrolled or life-threatening bleeding.[17] There is limited information available concerning their use in companion animals, mainly due to platelet transfusion product availability being a finite resource due to many factors.

The aim of this clinical trial is to assess the safety and efficacy of lyophilized platelets to ascertain if they have a role in managing and treating actively bleeding, severely thrombocytopenic canines.

Lyophilized platelets

Lyophilized Platelets have been developed through blood banking research over a 70 year period.[87] Multiple methodologies for lyophilization have been developed with varying functionality. Lyophilized platelets look to solve an unmet need for an effective primary haemostasis product that combines availability, functionality and improved product safety (when compared to products already available). In human medicine this is particularly important where standard platelet transfusions are in short supply, examples of which would be in areas of geographical isolation, or in the developing world. In the developed world examples of potential areas where this product may be considered important would be in actively bleeding platelet refractory patients, or pre-hospital treatment including military and disaster medicine use. A close parallel exists in the veterinary field, where platelet products are either not available, in short supply, or only available in forms that take time to prepare. Most of the platelet products that the veterinary community can obtain provide inadequate platelet numbers to constitute an appropriate dose in severe thrombocytopenia. Pre-hospital treatment is also still in its infancy and veterinary patients often present at referral institutions many hours after sustaining injury or into their critical illness progression, with severe presenting symptoms.

Lyophilized platelets have been developed for the human market, with extensive preclinical phased testing for safety and efficacy (appendix 1). A key initial pre-clinical large animal (in comparison to rodent small animal) research model in development was the canine.

A canine cardiopulmonary bypass model was used to measure vessel bleeding time in dogs who had been on bypass, who were experiencing the known complication of depletion of platelets while the blood circulates in the extracorporeal bypass system. The infusion of lyophilized platelets at the point of weaning off bypass, was shown to consistently and persistently lower vascular bleeding times in test versus control dogs.[92] In previous studies and within these pre-clinical models, the subtle differences in

platelets between species was considered significant and the use of species specific lyophilized platelets in large animal models became standard. The collection and manufacturing procedures have been developed according to the nuances of each species.

Subsequent to the conclusions drawn in all preclinical trial work, trehalose buffered lyophilized platelets are now making their way into progressively more clinical trials for both canine and human patients.

In humans, autologous and allogeneic trehalose buffered lyophilized platelets (Thrombosomes®) have been transfused in a phase 1 open label multicenter safety trial to three cohorts of eight mildly bleeding thrombocytopenic patients.[106] Phase 2 trials into actively bleeding thrombocytopenic patients are ongoing.

In order to investigate these products for clinical indications in veterinary patients, phase 2/3 clinical studies into lyophilized platelets in the canine have been performed. An initial study published in 2012 comparing fresh room temperature stored apheresis derived platelets to formaldehyde stabilized lyophilized platelets in thrombocytopenic canines concluded that lyophilized platelets were non inferior to fresh platelets and that the fast reconstitution and administration of lyophilized platelets may allow them to be an effective and practical haemostatic bridge in a patient with life-threatening haemorrhage.[20]

A more recent phase 2/3 veterinary clinical trial in severely thrombocytopenic, actively bleeding canines compared the used of DMSO cryopreserved platelet concentrate to trehalose buffered lyophilized platelets. This study concluded that lyophilized platelets were safe to administer in thrombocytopenic canines and demonstrated efficacy with significantly lower decrease in haematocrit and significantly lower DOGiBAT bleeding score in patients receiving lyophilized platelets compared to a current standard of care cryopreserved (frozen) platelets one hour post transfusion.[26]

Trehalose buffered lyophilized canine platelets have been available on general sale to the US veterinary market since September 2018 as StablePlate RX®.

Trehalose buffered lyophilized platelets retain morphologic and functional characteristics of circulating platelets. They are considered phenotypically to be in a partially activated state when rehydrated and infused, enhancing their ability and the speed that they localise to the site of haemorrhage and act as a primary haemostatic agent. Practically they offer unique logistical benefits as a readily available, shelf stable, simple to use infusion for the control of active haemorrhage where currently there are very limited options to actively treat this significant and critical presentation within veterinary practices in the UK.

Methods

UK legislation meant that trehalose buffered lyophilized platelets produced in the US, although in general sale to the US veterinary community from September 2018, required an application for Veterinary Medicine Directorate (VMD) certification in order to be used in a UK clinical trial.

Lyophilized platelets are considered to be a biopharmaceutical product by the VMD, so an Animal test certificate (ATC) was required. This was applied for in Mar 2019 and obtained on 2nd May 2019 – Appendix 2

Lyophilized platelet source and manufacture:

Platelet concentrate is sourced for canines by apheresis from closed colony hounds and beagles kept in USDA licensed, AAALAC accredited sites. All donors are Dog Erythrocyte Antigen (DEA) 1 and 7 typed and tested negative for anti-DEA plasma antibody to all known DEA types. These donors receive preventative care as a standard, full vaccination to canine distemper, parvovirus, infectious canine hepatitis, parainfluenza, bordatella bronchiseptica and leptospirosis. They are tested quarterly for

infectious diseases according to the consensus statement from the American College of Veterinary Internal medicine (ACVIM) on testing for blood-borne pathogens in canine and feline blood donors[96].

Apheresis derived platelet concentrate is obtained in a batch (18-20 apheresis units). All platelet units are leucoreduced. Canine Trehalose buffered lyophilized platelets (StablePlate RX®) are manufactured in a cGMP compliant environment with standard operating procedures (SOP's) for manufacture, quality control and quality assurance similar to that maintained in human biopharmaceutical production of Thrombosomes®, the trehalose buffered human lyophilized platelet product. The laboratory including the use of an ISO 7 clean room, operates under Good Laboratory practice (GLP) and Good Manufacturing practice (GMP) guidelines and has been reviewed by the FDA.

Platelets are lyophilized (freeze dried) to preserve their integrity and biological function. Platelets are stabilized using a method of cross linking with trehalose before lyophilization.

Trehalose is a naturally occurring sugar that acts as an energy source biosynthesized by plants, yeast, fungi, some insects and invertebrates where it is known to act to stabilize or protect the living cells against stress such as heat or desiccation in nature.

Trehalose buffer was used to preserve the platelets within the study test solution, the control solution for this study is freeze dried trehalose buffer solution identical to that used in the test solution but without the platelets added.

As part of a masters in veterinary research, through Edinburgh university, application for welfare and ethics approval was made via the veterinary ethics review committee (VERC)– University of Edinburgh in November 2018 and obtained on the 19th June 2019 - Appendix 3

To become part of a multicenter trial, the Royal Veterinary College (RVC) also required their own independent ethical review via their Clinical Research Ethical Review Board (CRERB) – this was submitted in November 2018 and granted on the 10th May 2019 - Appendix 4

To calculate the number of enrolments required for a study designed to look at both safety but also for efficacy, a power calculation was performed based on recognizing significant difference using formula 1. This was looking for statistically significant difference between the control and treatment group with respect to what was considered a clinically relevant change in DOGiBAT score (2)

With a Sample size of 16 (15.68) dogs per treatment group and a true mean difference in the primary endpoint DOGiBAT score of 2, there is 80% power to obtain a statistically significant difference between treatment groups at the 5% (p=0.05) significance level

4 additional dogs were added to each group (treatment and control) to try to produce an adequately powered study for efficacy determination. The additional dogs were to account for dropouts due to but not confined to mortality, withdrawal from the study and missing endpoint data.

Formula 1 – Power Calculation

No Samples per group (n) = $\frac{2\sigma^2(Z_{(1-\alpha/2)} + Z_\beta)^2}{\Delta^2}$

Where:

$Z_{1-\alpha/2}$ = normal deviate for α error (two-sided test uses $Z_{1-\alpha/2}$) (≈ 1.96, when $\alpha = 0.05$)

$Z_{1-\beta}$ = normal deviate for β error (≈ 0.84, when $\beta = 0.20$)

2 indicates two groups (sources of variability) i.e. treatment group; control group

Δ = least difference between treated group and control group that is considered clinically significant

n = sample size of each group

As thrombocytopenic patients are considered a relatively uncommon presentation in the UK, with a pre-trial survey of busy referral institutes estimating 1-2 cases per month at the most, the trial was designed as a multicenter trial incorporating 5 emergency and referral centres across the UK.

Centres and clinical leads

Vets Now Glasgow: Daniel Lewis MA VET MB Dip ACVECC cert VA MRCVS

RDSVS Hospital for small animals, Edinburgh: Craig Breheny BVM&S Dip ECVIM-CA MRCVS

Davies Veterinary Specialists, Hertfordshire: Patricia Ibarrola Dip ECVIM CA DSAM MRCVS

Dick White Referrals, Cambridgeshire: Simon Tappin MA VETMB cert SAM DipECVIM CA FRCVS

Royal Veterinary College, Hertfordshire: Karen Humm MA VETMB cert VA DipACVECC FHEA DipECVECC MRCVS

Site feasibility was determined through email, telephone consultation and face to face discussion. A face to face site initiation visit by the clinical study lead (JW) to each site's clinical lead, involving a powerpoint presentation (Appendix 5) that highlighted all the key areas of the trial and provided DOGiBAT training material was performed between October and November 2018. A complete clinical trial file containing all of the trial literature and consent forms, case logs, adverse event forms, randomization instructions via sealed envelope and budget terms was supplied in printed form. Clinical leads signed consent to comply with the detailed clinical trial protocol prior to the site initiation visit. Updated versions of literature have been provided throughout the trial to all clinical leads as required, with a central literature store sited with the clinical study lead. Comprehensive clinical trial details are contained within the detailed clinical protocol and the investigators brochure, prepared by the clinical study lead. Appendix 6 and 7

Twelve vials of control and test solution, enough for each centre to treat two 30kg dogs in each arm, faecal occult blood test strips and urinary test strips[a] were hand delivered or shipped by tracked courier to all 5 centres in the first two weeks of July 2019. A further batch of solution was required to fulfill the full study enrolment expectation and shipped from the US to the UK in March 2020.

The first enrolment occurred on 29th July 2019

Across the 5 centres there was 6 enrolments by January 2020, representing 1 case a month. In order to enroll the full case number in a timely manner, in February 2020 a change control to the VMD ATC was made to add a further 4 UK centres.

Additional centres and clinical leads- March 2020

Durham and Washington Vets4pets: Kevin Walton BVM&S MRCVS

Southern Counties Vet Specialists: Andrea DiBella Cert SAM DipECVIM-CA MRCVS

Linnaeus (Paragon Veterinary Specialists, Willows Veterinary Specialists):

Linnaeus lead: Sophie Adamantos BVSc CertVA DipACVECC DipECVECC MRCVS.

Paragon Veterinary Specialists: Andrea Holmes BVSc DipECVIM-CA MRCVS

Willows Veterinary Specialists: Ludivine Boiron MSC DVM DAVECC DiplACVECC MRCVS

[a] Urinary Test Strips: Siemens Multistix® 10 SG
Faecal occult Blood testing strips: Siemens Hema-Chek®Fecal Occult Blood Test
made in PL, Siemens Healthcare Diagnostics Inc 511 Benedict Avenue Tarrytown NY 10591-5097 USA

The criteria for the study required patients not only to be severely thrombocytopenic (platelet count of <50x 10^9/l) but also actively bleeding (DOGiBAT score of ≥2 on enrolment). DOGiBAT scoring is a published veterinary bleeding score system for thrombocytopenic dogs, that assesses bleeding in nine anatomical sites and provides a score of 0, 1 or 2 for each site, equating to no bleeding, mild bleeding or severe bleeding. The summation of the scores gives a score out of a total of 18.[64]

Inclusion and Exclusion criteria are illustrated in Table 2. No limitation was made on the aetiology of the thrombocytopenia. The trial would compare canines receiving standard medical management of their underlying condition receiving a lyophilized trehalose buffer solution (control) to canines receiving standard pharmacological management of their underlying condition plus trehalose buffered lyophilized platelets (test) – see clinical trial schematic below. Whether test or control solution was administered was blinded to the clinical leads and clinicians administering the solutions at each establishment.

To represent standard pharmacological treatment regimes for disease processes underlying the thrombocytopenia, specific "rescue" interventions for enrolled patients were allowed after T1. Centres were also aware that they could withdraw a patient from the study at any time, should they feel the deviation was required on a patient welfare basis. Specifically, vincristine and packed red blood cells were allowed at the discretion of the treating clinician after the T1 (one hour post administration of test or control solution) timepoint to represent standard medical management of deteriorating clinical cases that were not responding to first line treatment.

Tranexamic acid, cyclophosphamide and Intravenous Immunoglobulins were not allowed in the 72 hours preceding enrolment, so administration in this timeline precluded enrolment. In enrolled patients they were not allowed to be administered until after the T24 (24 hours post administration of test or control solution) timepoint.

Inclusion Criteria at enrolment
DOGiBAT score of >2
Platelet count of <50x10^9/l
APTT/PT normal (+/-20% of reference range)
Weight within the range of 2-45Kg
Age >9 months
Permitted drug treatments: Glucocorticoids, NSAIDS, antibiotics, cyclosporine, mycophenylate. antacids, anxiolytic medication, analgesics, sedatives, general anaesthesetic drugs, intravenous fluids (crystalloids) and antiemetics.
Exclusion criteria at enrolment:
Transfusion with any platelet containing blood products within the preceding 72 hours, this includes stored or fresh whole blood, packed red blood cells and plasma products.
Patients currently treated for CHF or primary hypertension
Patients that have undergone surgery within the preceding 72 hours
Administration of Tranexamic acid, cyclophosphamide, vincristine and intravenous immunoglobulins within the preceding 72 hours

Table 2: Clinical trial inclusion and exclusion criteria

Control and test solution were labelled solution A and B for blinding purposes. Control and test solution shipped from the US in June 2019. On arrival they were inventoried and placed in a centralized temperature monitored pharmacy.

Study interventions:

Once enrolment inclusion and exclusion criteria were confirmed and owner consent obtained, patients were randomized to receive test solution or control solution.

Randomization was performed via an online platform – https://www.sealedenvelope.com . This allocated each patient to receive either solution A or solution B.

An appropriate dose of the allocated solution was reconstituted according to patient bodyweight. 1.6ml/kg of reconstituted solution was administered intravenously at a recommended rate of 1ml/minute – no in line filter was required and syringe drivers could be used. Patients were closely monitored during the infusion, and any adverse effects were to be recorded using a comprehensive adverse reaction form (within appendix 6). Any remaining solution was discarded as clinical waste.

Blinding clinicians was possible due to the ability to formulate a control buffer solution that presented in the same way, reconstituted in the same manner and was visually, virtually indistinguishable from the lyophilized platelet solution.

For the purpose of blinding, test product vials and control product vials were labelled either solution A or solution B and the label also contained the wording, for use in the (Animal test certificate) ATC licensed clinical trial only, to comply with ATC trial regulation. Each individual vial was identified with a 13 digit batch and lot allocation code containing a 3 digit identification number unique to each vial. The vial contents were visually indistinguishable from each other prior to rehydration . They were presented as a pelleted off white powder in a sealed 50ml vial. Reconstitution for a 5kg dose was with 8ml of

water for injection immediately prior to administration. Detailed rehydration and administration instructions were provided with each batch of trial vials delivered to clinical sites. There was an additional copy within the detailed protocol and investigators brochure provided to each centre (Appendix 6 and 7).

The test solution has a final particulate concentration of 1.5 x 10^{10} platelets per 5kg dose.

Both test and control solution contained the following excipients: HEPES, NaCl, KCl, Dextrose, NaHCO3, trehalose, ethanol and polysucrose.

Patient data collection

Enrolled patients were evaluated to include a platelet count, haematocrit, PT, APTT and DOGiBAT score prior to administration (T0) , one hour post finishing administration (T1) and twenty four hours post finishing administration (T24). Biochemistry was performed at T0 and T24.

DOGiBAT score sheets for the three timepoints were provided to facilitate standardisation of scoring (within Appendix 6).

1 and 2 week follow up post enrolment was performed, by telephone should the patient have been discharged, by the clinician in charge of the case.

Biochemistry, Haematology and Coagulation analysers:

As a nine site study and with a 24 hour study period, a variety of internal validated laboratory machines and external reference laboratories were used for analyses. Where possible the same analysers were used across the timepoints. In house analysers were Idexx Catalyst Dx, Procyte Dx, Coag dx, Woodley Q labs insight coagulation machine, Beckman Coulter AU480 and Sysmex XT 2000ii. Numerical analytical results were recorded in the same units and collated centrally by the study lead. Access to manual

methods for haematocrit and platelet count to validate machine results were also confirmed as present at each clinical site.

Primary and secondary endpoints were set. Three primary endpoints of DOGiBAT score, haematocrit and platelet count and three secondary endpoints of all-cause mortality, length of hospitalization and need for additional transfusion support. Primary endpoints were to be measured and analysed at three timepoints, enrolment (t0), one hour post finishing infusion of trial solution (t1) and twenty-four hours post finishing infusion of trial infusion (t24). Mortality and additional transfusion support were to be recorded over the 24 hour trial period and also within the hospitalization period. Length of hospitalization was to be recorded from the day of enrolment to the day of discharge.

Statistical analysis

Database management: Data was stored in an Excel spreadsheet with patient ID as the main key identifier. Prior to analysis all variables were examined. Platelet counts less than the detection limit were given a value of ½ the limit of detection (i.e. <5, 2.5; <3, 1.5). Platelet values were then log10(x+1) transformed to correct for non-normal distribution prior to analysis. For some animals, respiratory rate was recorded as “pant”. This was defined as 30. Sex, neutered animals grouped with non neutered animals of the same gender (i.e. F=F+FN; M=M+MN). Sites other than Glasgow, Davies or Willows were grouped and defined as “other”. Age at enrolment (years) was defined as (date of enrolment-patient date of birth)/365)

Baseline differences between test and treatment groups for categorical variables was performed using Fisher’s exact text using StatXact version 11 (Cytel MA, USA). Continuous variables were analysed using T-test although the variables were also analysed using a Mann-Whitney as the sample size was too small to accurately assess the normality of the data. The results were comparable.

Analysis of the differences in pre and post transfusion DogiBAT, HCT and platelet results were performed using a General Linear Mixed Model. The model was run using group (treatment, control) and time (T0, T1, T24) as fixed effects in the model. Patient ID was included as a random term in the model. The interaction was tested for all models but it was not significant.

Unless stated otherwise all statistical analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC). Statistical significance set at $p<0.05$.

Safety analysis

Safety Analysis was based on monitoring for adverse events. Patients were monitored directly during the control and test solution administration. Any adverse event within the study monitoring period was to be notified to the centre clinical lead, and if considered associated with the administration of trial solution was to be immediately notified to the principal lead. In addition all fatalities (death or euthanasia) were to be notified to the principal lead and evaluated for possible relation to product administration. A safety analysis report was to be generated at interim (50%) enrolment, and assessed by an independent data analysis committee, prior to continuing to full enrolment.

Efficacy analysis

Efficacy analysis was to be based on demonstrating a significant difference between test and control groups in Primary endpoints. Efficacy would be considered demonstrated should significant ($p<0.05$) difference between groups in stabilization in haematocrit, increase in platelet count or stabilization and improvement in DOGiBAT score be seen. Secondary endpoints analysis of all-cause mortality, length of hospitalization and need for additional transfusion support were to be explored in relation to efficacy. Statistical significance was not expected in these secondary endpoints in this trial with the limitation to

numbers, this limitation being decided for pragmatic reasons of enrolment rates, realistic study length and costs.

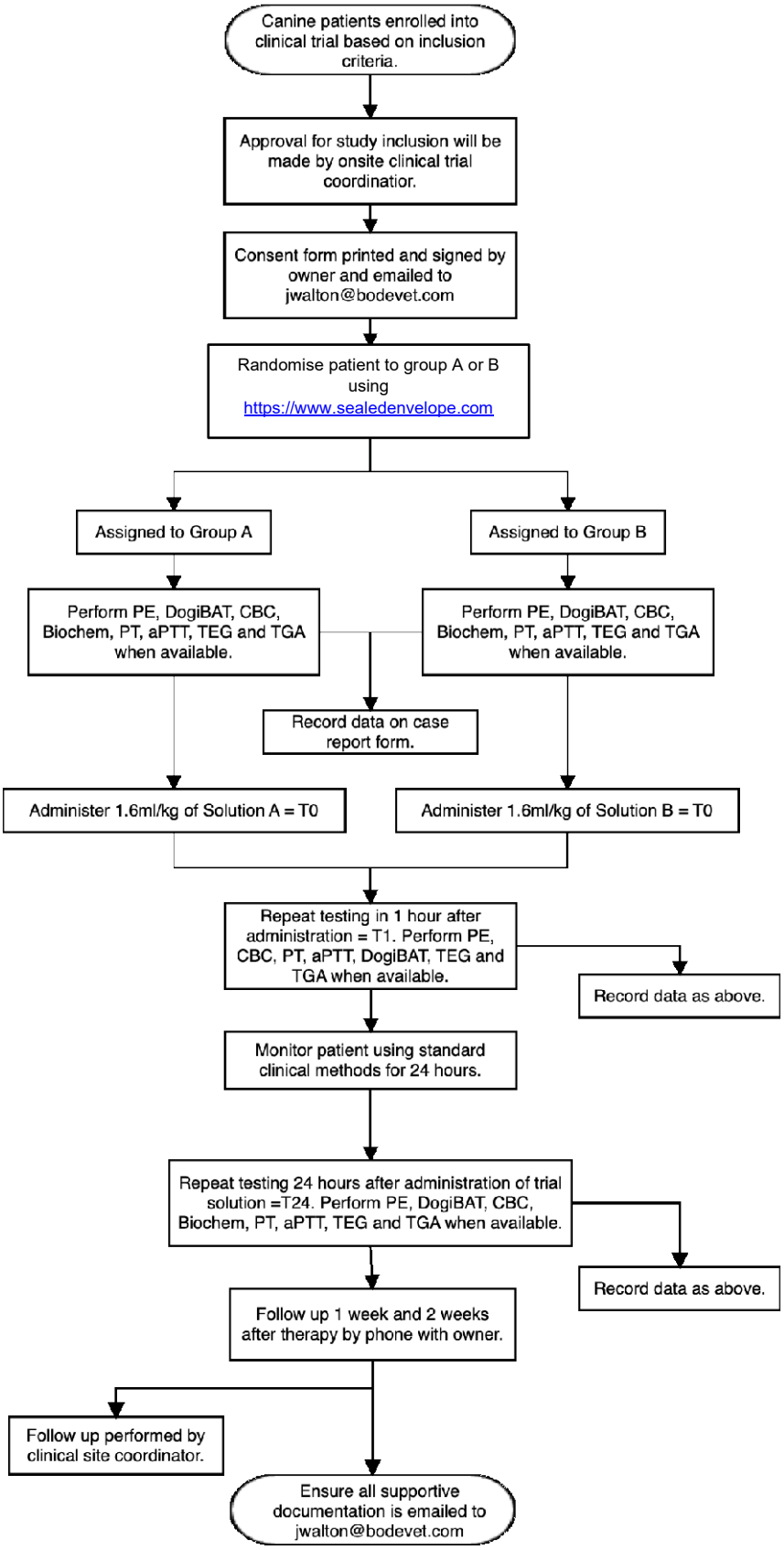
Clinical trial synopsis

TITLE	**Evaluation of stable plate Rx® in thrombocytopenic canine patients: a multicentre clinical trial**
RESEARCH INSTITUTE, LEAD AND SUPERVISORS.	Edinburgh University – Via MSc in Clinical research. Research Lead – Dr Jenny Walton BVM&S MRCVS. MSc Supervisors - Professor Richard Mellanby BSc BVMS PhD DSAM DipECVIM-CA FRCVS Simon Tappin MA VetMB CertSAM DipECVIM-CA FRCVS
FUNDING ORGANIZATION	Bodevet, Inc
NUMBER OF SITES	9
RATIONALE	Small Animal Referral, Emergency and Critical care centres and University hospitals involved in emergency medicine are currently managing hemorrhage due to primary hemostatic defects using drug treatment alongside fresh whole blood – very occasionally Platelet Rich Plasma is harvested from whole blood donations. Most hemorrhage is managed without transfused platelets due to lack of availability of a UK banked platelet product and the challenges involved with harvesting appropriate volumes of fresh whole blood. Single case reports and small review studies suggest that platelet transfusion is preferable over whole blood administration. Preclinical studies suggest that Stable Plate Rx® is safe in the dog at multiple dosing ranges. A previous clinical study evaluated a lyophilized platelet product suggesting a role in management of haemorrhage caused by thrombocytopenia.
STUDY DESIGN	This is a multicenter, blinded, randomized safety and efficacy study.
PRIMARY OBJECTIVE	To evaluate the safety of StablePlate RX ® in clinical canine patients for the control of life threatening haemorrhage secondary to thrombocytopenia.
SECONDARY OBJECTIVES	To evaluate the efficacy of StablePlate RX® in clinical canine patients demonstrating life threatening haemorrhage secondary to thrombocytopenia.
SUBJECT SELECTION CRITERIA	Inclusion Criteria: Canine patients will be evaluated and selected using a standardized bleeding assessment tool, DOGiBAT. This assessment tool can be utilized to assess bleeding in nine anatomical sites with a grade of 0(none) to 2(severe). Dogs must score a “2” in one area or a “1” in two or more areas to

	be included in the study. Patients must also have a platelet count of 50,000/ul or less for inclusion. Exclusion Criteria: Patients may not receive platelet containing blood products within the 72 hours before enrolment into the study. Patients must have a hematocrit >20% at the time of enrolment into the study. Patients must be greater than 2kg and less than 45kg. Patients currently treated for CHF or primary hypertension at the time of enrolment into the study will be excluded. Patients must demonstrate a normal PT and aPTT at the time of enrolment into the study. Patients that have undergone surgery within the preceding 48 hours of enrolment will be excluded.
EXPERIMENT GROUP	Clinician led standard medical management that has been initiated no more than 48 hrs prior to time 0 StablePlate RX ®, the lyophilized platelet product (LCP) will be administered by single intravenous bolus injection at a dose of 3.0 x 10^9LCP/kg which equates to 1.6 mls of solution/kg at time 0
CONTROL GROUP	Clinician led standard medical management that has been initiated no more than 48 hrs prior to time 0 1.6 mls/kg of a trehalose buffer solution will be administered by single intravenous bolus injection at time 0
CONCOMMITANT MEDICATIONS	Allowed: Steroid (intravenous, oral), cyclosporine (intravenous, oral), antibiotics, mycophenylate Allowed after T1: Vincristine and Blood Products Allowed only after T24: Cyclophosphamide, IVIgG (human or canine) Tranexamic acid (TXA)
Efficacy Evaluations	Patients will be evaluated prior to administration, 1hour post-administration and 24 hours after administration by automated CBC, PT, APTT, and complete

	physical examination including DOGiBAT scoring. When available by location TEG and TGA evaluations may be assessed prior to administration, immediately post administration 1hour post administration and 24 hr post administration
Safety Evaluations	CBC, Biochemistry, PT, APTT, and complete physical examination including DOGiBAT scoring at zero timepoint, 1hour post-administration (excluding biochemistry) and 24 hours post-administration (including biochemistry if considered by the clinician to be clinically relevant)
Planned Interim Analyses	When approximately 50% of subjects have completed the study, an interim analysis for safety will be conducted by an independent data monitoring committee. Serious adverse events will be monitored by the study coordinator on an ongoing basis throughout the study.

Clinical trial schematic



Interim trial analyses and discussion

Results

Twenty dogs were enrolled, 14 neutered females, 1 entire female, 4 neutered males and 1 entire male. Breeds represented, 5 crossbreeds (including two designated Cockapoo and one designated Labradoodle), 3 English Springer Spaniels, 2 Hungarian Vislas, 2 Miniature Schnauzer, 1 Cocker Spaniel, 1 Cavalier King Charles Spaniel, 1 Old English Sheepdog, 1 Bichon Frise, 1 German Shepherd, 1 Basset Hound, 1 Pug and 1 Lhasa Apso. Mean age 6 years (1-11yrs). Diagnoses of immune aetiology either primary ITP (16), secondary ITP (2) or combined IMHA and ITP (1) was made for all but one case. No cases were confirmed as ITP based on antiplatelet antibody, as this test is not available in the UK. Of the secondary ITP cases, one was secondary to Ehrlichiosis, one was suspected possibly to be associated to nodular mammary neoplasia. One case was thrombocytopenic associated with pancytopenia and sepsis. Of the four non primary ITP cases, 2 were in the test group and 2 were in the control group. Medical management included steroid (n=18), vincristine (n= 10), proton pump inhibitors (n=13), maropitant (n=8), IVFT (n=11), antibiotics (n=5).

Five dogs in total received red cell transfusions. Three dogs required packed red cell rescue intervention in the 24-hour study period between t1 and t24 and two patients received packed red cells within the period of hospitalisation (both day 3), two of the rescue interventions between t1 and t24 were within the control group, 1 rescue intervention between t1 and t24 was in the test group as were the two cases that required red cells on day 3.

The overall mortality across the groups within the 24-hour study period was 3 (15%) and during the period of hospitalisation 5 dogs died (25%). The mortalities within the 24-hour endpoint study period equated to two in the control group and one in the test group. One control group dog was euthanised

at T1 due to ongoing and worsening signs of pulmonary haemorrhage, one control group dog which was the one case that had thrombocytopenia associated with pancytopenia and sepsis died at T18 due to suspected worsening GI bleeding and sepsis. One test group dog died of suspected Intracranial haemorrhage at T14, this dog was jaundiced on enrolment, suspected to have Evans' syndrome and had the highest entrance DOGiBAT score in the study (10).

Hospitalisation times to discharge were available for 15 patients. Mean hospitalisation time in the test group was 4.375 days (1-7) and in the control group 4.571 days (2-8)

Baseline parameter analysis

With twenty enrolments there was no significant differences between groups for entrance criteria sex, weight and diagnoses (Table 3) or in clinical parameters of respiratory rate, heart rate and temperature.

Primary endpoint analysis

Primary endpoint results (median and ranges) in each group (Test and Control) at all three timepoints are illustrated in Table 1. In this interim analysis there was no significant difference between groups at any timepoint or within groups over time (Figures 2-10) The study is powered to detect significance at 40 enrolments, so the lack of significant difference is not unexpected at this time with only 20 enrolments.

It was noted that mean entrance DOGiBAT score in the test group was 7 (3-10) compared to the control group 4 (3-9), though the ranges overlap

It was considered appropriate to perform limited post hoc subgroup analysis on the interim data due to the small number of enrolments and heterogenous patient population. This subgroup was defined by removing patients that had missing endpoint data through mortality (three patients), or had received a

rescue red cell transfusion between T1 and T24 (three patients, but one was also a mortality in 24 hours) This subgroup data from the fifteen remaining patients is illustrated in tables 5 and 6.

Secondary endpoint analysis.

In this interim analysis there was no significant difference between groups in secondary endpoints of mortality, length of hospitalisation or need for additional transfusion.

Time	Group	DogiBat	Platelets	HCT %
T0	Test	7.0 (3.0-10.0)	4.0 (0-22.0)	35 (21-57)
	Control	4.0 (3.0-9.0)	2.0 (0-43.0)	35 (22-50)
T1	Test	7.0 (2.0-10.0)	0.0 (0-36.0)	31 (19-56)
	Control	4.5 (1.0-7.0)	0.75 (0-29.0)	33 (19-47)
T24	Test	5.5 (2.0-8.0)	1.0 (0-233.0)	28 (20-60)
	Control	4.0 (1.0-8.0)	1.0 (0-39.0)	38 (24-45)

Table 3: All Primary endpoint results of DOGiBAT score, HCT % and Platelet count ($x10^9$/l) (medians and ranges) for Test and Control Group at all three timepoints (T0,T1 and T24)

	Test	Control	**Fisher's exact Test statistic**	**P-value**
Sex			**0.1555**	**1.00**
F+FN	8	7		
M+MN	3	2		
Site			**4.707**	**0.2332**
Davies	1	4		
Glasgow	5	3		
Willows	2	2		
Other (Edinburgh + Southern counties + Vets4Pets)	3	0		
Diagnosis			**0.7371**	**0.5658**
ITP	11	8		
Other	0	1		

Table 4: Categorical Variables - Sex, enrolment site and diagnosis.

Fishers exact testtest comparison between groups Test vs Control and calculated p value. Statistical significance was set at a p value of <0.05. There was no statistically significant differences in categorical variables between groups

Group	Enrolment DOGiBAT	Enrolment Hct T0 %	Hct T1 %	Hct T24 %	Change between T0 and T1 %	Change Between T1 and T24 %	Change between T0 and T24 %
Test	4	57	56	60	-1	+4	+3
Test	7	35	35	34	0	-1	-1
Test	4	34	40	34	+6	-6	0
Test	3	35	36	31	+1	-5	-4
Test	7	22	23	26	+1	+3	+4
Test	7	34	31	24	-3	-7	-10
Test	3	33	25	28	-8	+3	-5
Test	7	21	20	20	-1	0	-1
Test	8	36	30	21	-6	-9	-15
Test	6	38	36	27	-2	-9	-11
Test	10	43	30	n/a			
MEAN	5.6	34.1	32.9	30.9	--1.2	-2	-3.2

Table 5 : Enrolment DOGiBAT and Haematocrit (%) difference between time points Test group. Data removed from calculations for patients who did not have 3 complete timepoint results or received rescue red cell intervention

Selected endpoint data for 11 dogs in the test group over the 24 hour study period. DOGiBAT entrance scores and HCT changes over the three time points T0, T1 and T24. Difference in HCT between timepoints calculated and mean difference within the group shown. 1 dog, highlighted blue removed from mean calculations, due to mortality and missing 3 point data (no t24 result), and one dog highlighted red, removed from mean calculations due to rescue red cell transfusion between t1 and t24

Group	Enrolment DOGiBAT	Enrolment Hct T0 %	Hct t1 %	Hct 24 %	Change between T0 and T1 %	Change Between T1 and T24 %	Change between T0 and T24 %
Control	5	50	36	39	-14	+3	-11
Control	3	36	27	36	-9	+9	0
Control	4	50	47	45	-3	-2	-5
Control	5	35	30	33	-5	+3	-2
Control	4	22	20	24	-2	+4	+2
Control	5	41	39	43	-2	+4	+2
Control	5	35	36	n/a			
Control	9	32	n/a	n/a			
Control	4	25	19	33			
MEAN	4.3	39	33	37	-5.8	+3.5	-2.3

Table 6: Enrolment DOGiBAT and Haematocrit (%) difference between time points Control group. Data removed from calculations for patients who did not have 3 complete timepoint results or received rescue red cell intervention

Selected Endpoint data for 9 dogs in the control group over the 24 hour study period. DOGiBAT entrance scores and HCT (%) changes over the three time points T0, T1 and T24. Difference in HCT between timepoints calculated and mean difference within the group shown. 3 dogs removed from mean calculations, 1 due to mortality (highlighted blue), one due to rescue red cell transfusion between t1 and t24 (highlighted red) and one due to mortality and rescue red cell transfusion between t1 and t24 (highlighted red and blue)

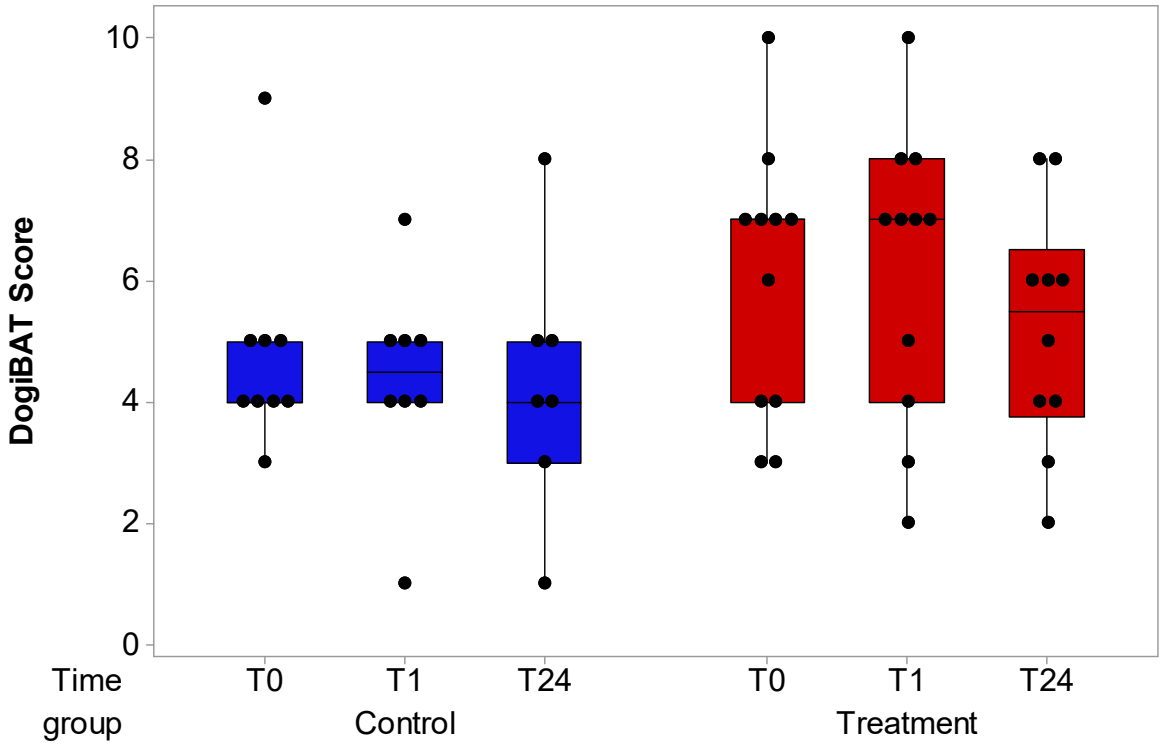


Figure 2: Box and Whisker Plots representing the change in DOGiBAT score in all dogs over time. Illustrating test and control groups across all three time points(T0, T1, T24). All Data. Individual dots represent the data points. The box represents the interquartile range with the median represented as a line.

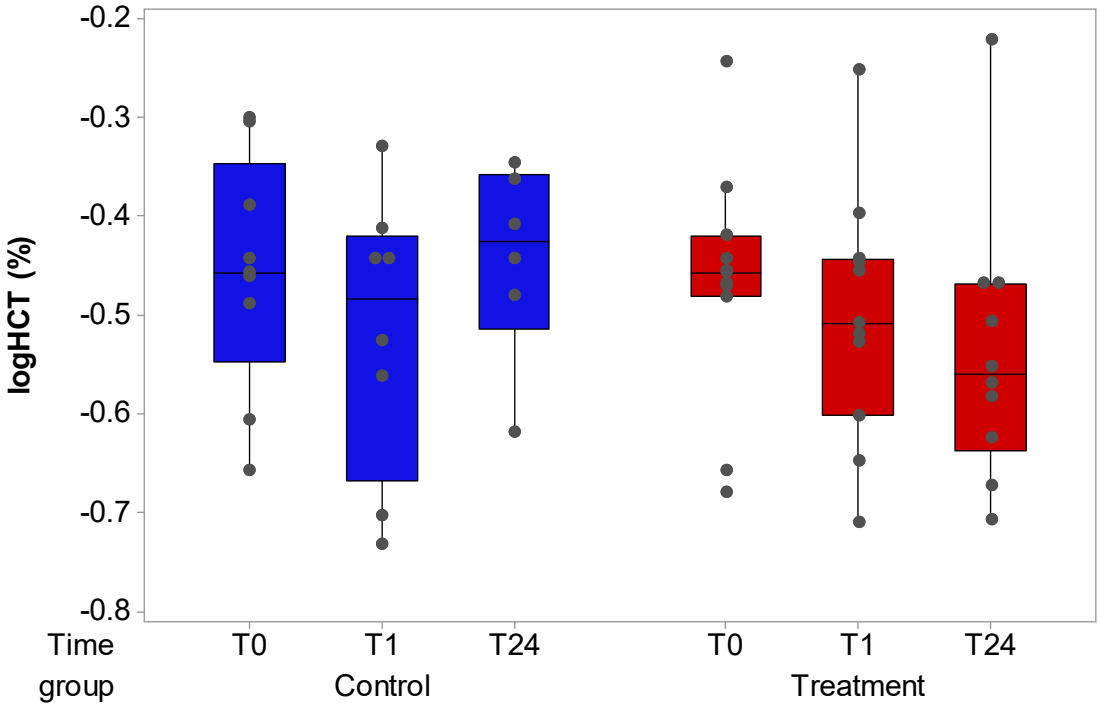


Figure 3: Box and Whisker Plot representing the change in logHaematocrit (logHct) in all dogs over time. Illustrating test and control groups across all three time points(T0, T1, T24). All Data. Individual dots represent the data points. The box represents the interquartile range with the median represented as a line.

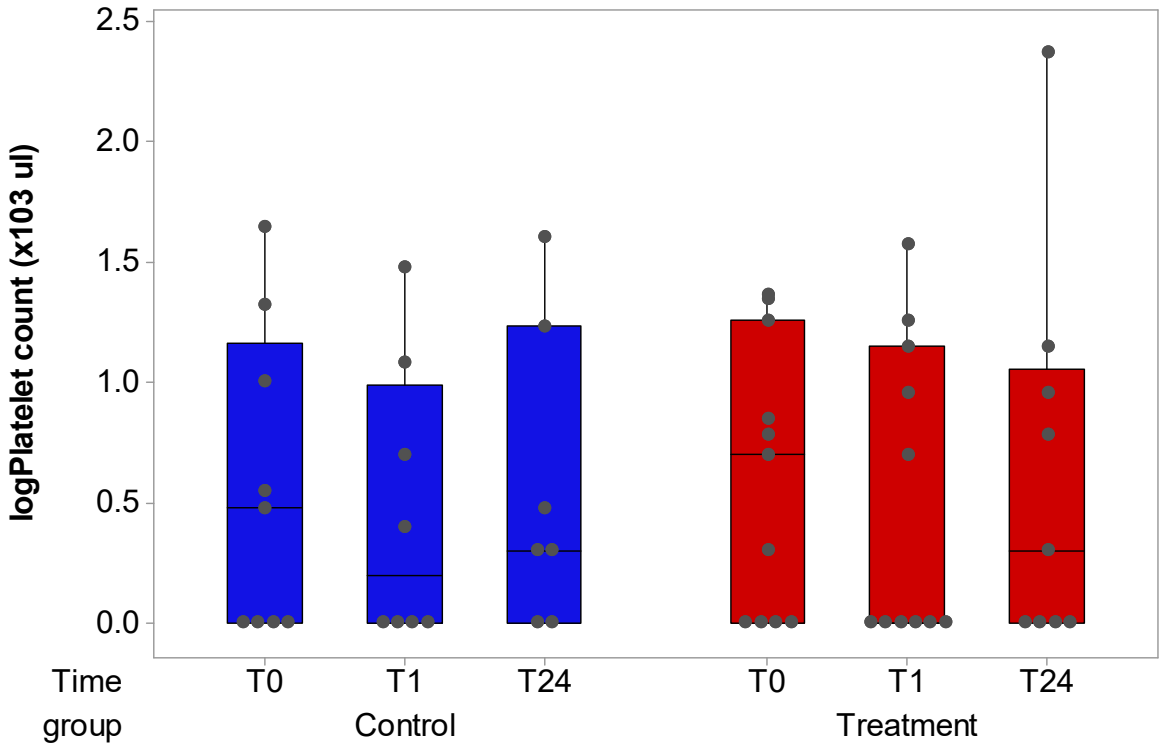
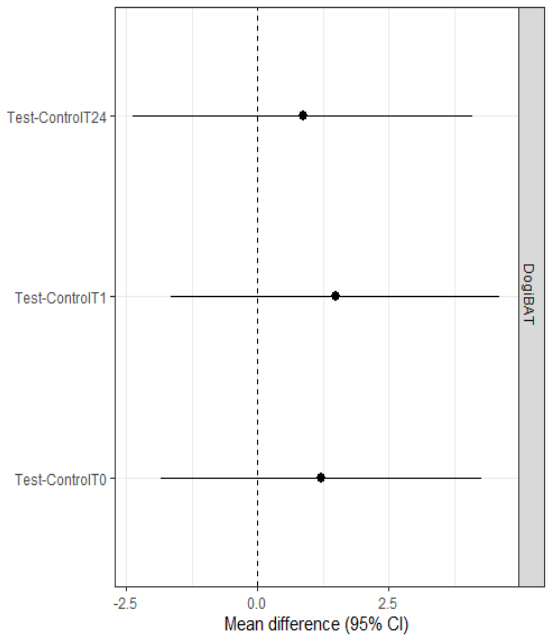
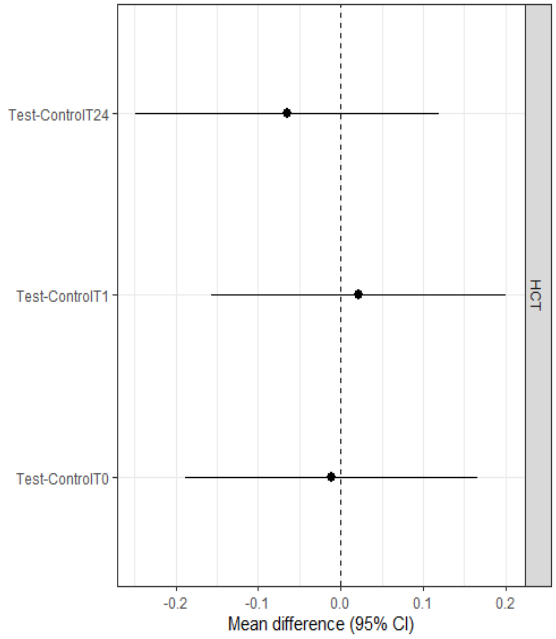


Figure 4: Box and Whisker Plot representing the change in logPlatelet count in all dogs over time. Illustrating test and control groups across all three time points(T0, T1, T24). All Data. Individual dots represent the data points. The box represents the interquartile range with the median represented as a line.





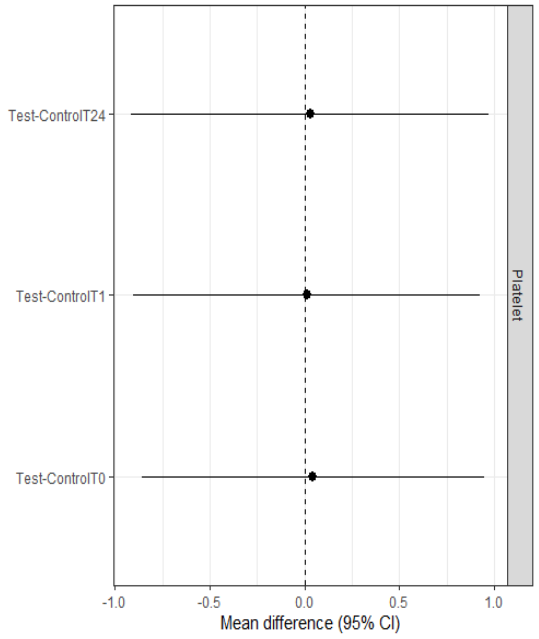


Figure 5 Figure 6 Figure 7

Figures 5 to 7:: Represent the mean difference between Test and Control Group at each of the timepoints T0, T1 and T24 for DOGiBAT score (figure 5), Haematocrit (figure 6) and Platelet count (figure 7)

The dot represents the mean difference and the horizontal whisker line represents the 95% confidence interval of the mean difference. The vertical zero line indicates no difference between the two groups for the variable measured.

The figures illustrate no significance in the mean difference between groups in primary endpoints at T0, T1 and T24

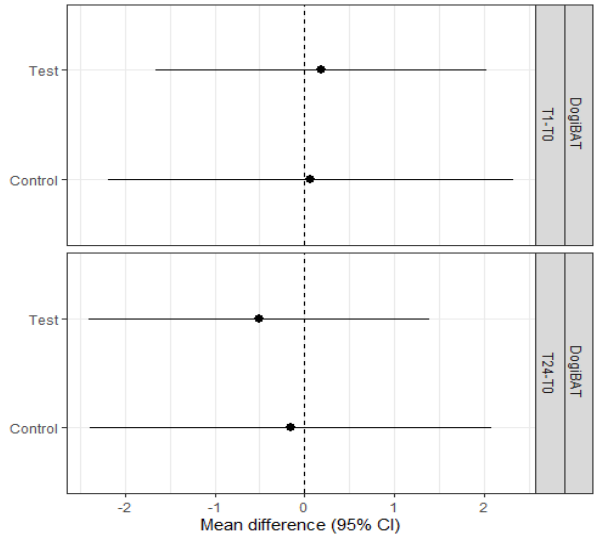


Figure 8

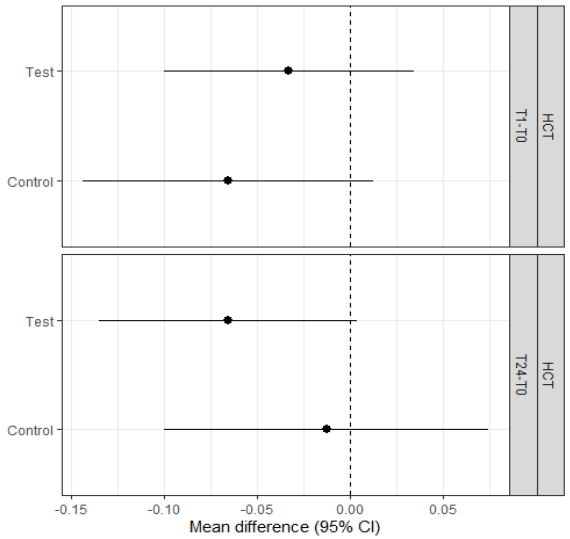


Figure 9

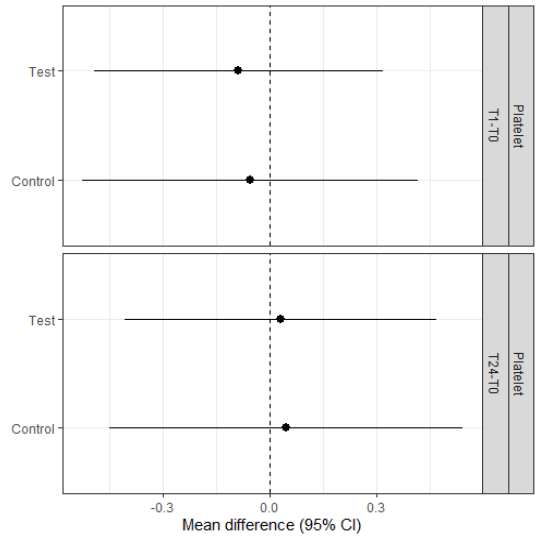


Figure 10

Figures 8 to 10: Represent the difference in DOGiBAT, (Figure 8) Haematocrit (Figure 9) , Platelet count (Figure 10), between different time points, T1-T0 and T24-T0 within test and control group.

The dot represents the mean difference, the horizontal whisker line the 95% confidence interval. The vertical zero line indicates no difference for the variable measured.

The figures illustrate no significance in the mean difference between time points within test or control group.

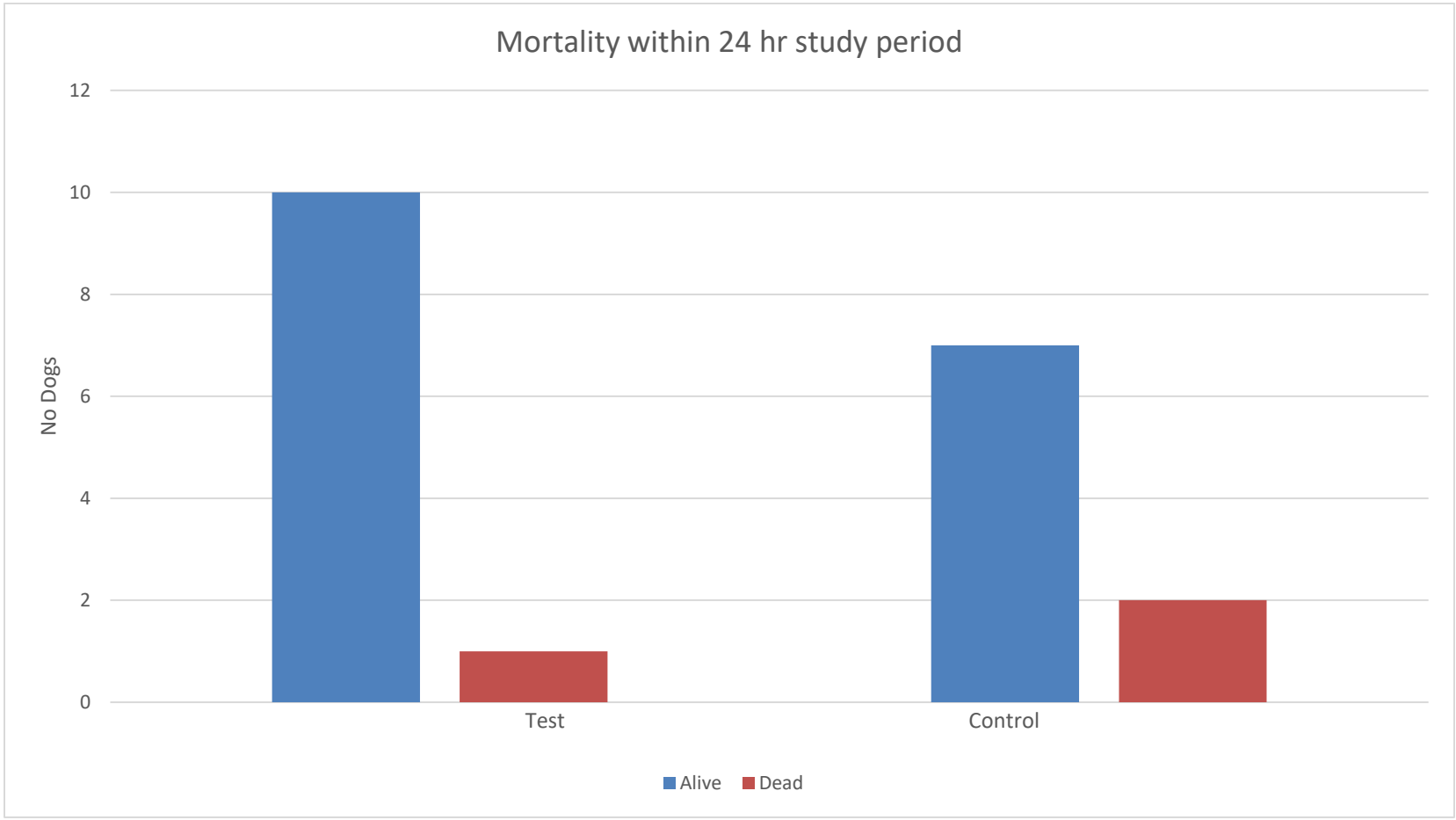


Figure 11: All-cause mortality within 24-hour study period test versus control group

There was no statistical difference demonstrated between test and control groups in terms of mortality in the 24 hour study period.

Discussion

This multicentre clinical trial across nine centres, designed as a randomised blinded controlled trial, has been an ambitious undertaking. Within the two year masters timeline half of the proposed enrolments have been made. The full enrolment number of 40 is designed to show significance in a clinically relevant endpoint, so it is not unexpected that currently no significant difference is seen. Insights into safety have been made, but significant clinical efficacy claims will take longer to define. Ongoing patient recruitment should allow further conclusions to be drawn.

In human medicine, severe thrombocytopenia with bleeding is treated with platelet transfusions. Prior to the availability of platelet only transfusions, mortality rates in leukaemia patients due to haemorrhage associated with thrombocytopenia was 63%. With the introduction of platelet transfusions, this reduced to 15%.[68]

There are many pathologies associated with thrombocytopenia and an exact comparison to the leukaemia-associated thrombocytopenias seen in humans is not possible at this current time in canine patients but indicates that platelet transfusions have found a very important place within human medicine.

This study was designed to recruit clinical cases with thrombocytopenia and active bleeding that were severe enough to fall into the category in human comparative medicine that would be considered for management with platelet transfusion.

Although ideally this masters project would have concluded with full study enrolment, interim results only are available at this point.

In a phase 2/3 clinical trial, criteria has to be strict in order to investigate both safety and efficacy between test and control groups. Bleeding thrombocytopenic cases that fitted the study criteria were slow to recruit. Nine UK centres, 8 of which were busy referral settings, took over a year to recruit half the proposed case number despite very committed clinical leads. Individual site clinical leads communicated regularly with the study lead and reported cases that narrowly missed inclusion for reasons such as out of criteria age, weight, coagulation times or Hct, the necessity for immediate packed red cell administration on presentation, surgery prior to presentation and also platelet counts of zero, but a <2 bleeding score. It may also be expected that some owners would also not feel obliged to consent to being involved in this phase of a clinical trial when considering their seriously ill pet. This challenge in recruitment has also been demonstrated by other veterinary multicentre studies incorporating busy referral and emergency clinics looking to recruit such cases.[20,56] The ability to recruit adequate numbers of cases within tightly controlled criteria to prospective studies may be something that is encountered when designing and carrying out veterinary studies at this level.

The UK has no available platelet transfusion option above fresh whole blood, which is not available as an immediate treatment option at any of the centres enrolling patients. This study is the first study of its kind able to compare two groups where one group receives a platelet transfusion option versus the other group medical management alone. This is also the first veterinary study with blinded treating clinicians investigating the efficacy of platelet products. This design should enable an unbiased investigation into the use of lyophilized platelets in the treatment of bleeding canine thrombocytopenic cases. The study was challenging to design, but the focus has always been on ensuring that the clinical trial was the highest quality research that could be performed. The steps above enabled the study to be carried out as a prospective, blinded, randomised clinical trial which is not commonly achieved in veterinary medicine at this time.[105]

When severe thrombocytopenic cases with signs of active bleeding present in canines, the most common aetiology presented both in this and previous trials is immune thrombocytopenia.[19,20] Nineteen out of twenty of the patients enrolled at this interim analysis stage for this study were considered to have Immune thrombocytopenia of either primary or secondary cause.

Immune thrombocytopenia, from the small volume of peer reviewed literature available, appears to be more common in dogs than it is in people. It is associated with more severe bleeding complications and a much higher mortality rate of 3-48% in canines vs 0.1-5% in humans. [29,47,53,55–59,63,79] There may be many factors involved that make the cross species comparison difficult. However, it is considered that there may be a role for platelet transfusion in canine thrombocytopenia cases to acutely control bleeding complications and stabilize patients through diagnostics and initiation of appropriate treatment. Active bleeding alongside severe thrombocytopenia in the human literature is one area that would indicate that platelet transfusions should be considered.[14]

The twenty dogs enrolled were severely thrombocytopenic and on average bleeding from at least three anatomical locations. The interim statistical analysis of all data shows no statistically significant differences in primary endpoints; so no efficacy conclusions can be drawn (Figures 2-10). However, subsequent to removing those dogs with missing endpoint data due to mortality or rescue red cell transfusions (Tables 5 and 6), there were trends seen when comparing test and control groups.

In this subgroup analysis, there was a mild decrease in DOGiBAT (less bleeding) in the test group compared to the control group, little change in the platelet count for both groups with a marginal increase in both groups, and for HCT, a mean decrease of 3.2% in the test group (1.2% between t0 and t1, and a further 2% between t1 and t24), compared to 2.3% in the control group (5.8% between T0 an T1 and a recovery of 3.5% between t1 and t24) – Tables 5 and 6

There are three canine studies within the last 10 years of literature that allow some comparisons to the current study be made, and allow some exploration within this discussion while at this interim point in enrolment. The comparisons are either due to similarities in enrolment criteria, use of platelet transfusions, similar endpoints or analysis of canine ITP patients. An overview of the comparable endpoint results from these studies is in Table 7.

The Goggs study illustrates the administration of cryopreserved platelets vs trehalose buffered lyophilized platelets, enrolling 88 bleeding thrombocytopenic dogs.[26] The Davidow study illustrates fresh platelet concentrate vs formaldehyde stabilized lyophilized platelets,[20] enrolling 37 dogs. The Levine Study explores an experimentally induced canine ITP model in 5 dogs over 24 hours, and provides nine naturally occurring thrombocytopenic cases as a comparison.[62]

The mean pattern seen across all patients over 24 hrs in the Goggs and Davidow trials, with platelet treatment alongside medical management, was a decrease in bleeding score (clinical improvement/reduction in active bleeding), mild increase but persistent thrombocytopenia <50x 10^9/l and a decrease in HCT of 4-6% . Both studies by their nature were unblinded, but both concluded non inferiority of the lyophilized platelets. In the Levine study, experimentally induced ITP canine model, that explored the progression of five dogs over 24 hours, there was an increase in bleeding score (increase in active bleeding) and a decrease in HCT of 7.9%.

In addition, the Goggs study reported superiority of trehalose buffered lyophilized platelets over DMSO cryopreserved platelets at decreasing bleeding and stabilizing haematocrit at the T0-T1 timeframe. The interim result trend in this UK study illustrates a larger drop in HCT in the control group 5.8% compared to the test group 1.2% between t0-t1 (table 5 and 6) despite higher mean entrance bleeding score in the test group. This may indicate that this could be a timeframe of interest concerning efficacy once the study enrols further cases.

When considering efficacy of platelet transfusions and one of the efficacy endpoints of this study, platelet transfusions are considered effective in human medicine when there is an expected rise in platelet count in the recipient, according to the dose administered. This has been the standard manner of assessing efficacy for many years, and appears sensible and reliable in many of the human thrombocytopenic conditions that require platelet transfusion.[107] Significant platelet count increase has not been seen in any of the canine studies illustrated in this discussion, and that is regardless of the platelet transfusion option used (lyophilized, DMSO cryopreserved, fresh platelets). Lack of increase in platelet count is also noted as a challenge in immune thrombocytopenia cases in humans that receive platelet transfusions, giving rise to questions of the efficacy of transfusions in this subset of patients. As the majority of canines enrolled in this and previous thrombocytopenia studies are ITP cases, extrapolated, this may mean that the same questions over efficacy arise. It is not standard practice to consider improvement in bleeding score or stabilization of haematocrit as an indicator of efficacy, though this is an area of ongoing consideration and focus in recent human literature.[74] This point may also have importance when considering a product with the circulation kinetics that trehalose buffered lyophilized platelets have been shown to have, when examined in preclinical studies. Lyophilized platelets are of an activated platelet phenotype, they are infused, target the site of haemorrhage and take part in primary haemostasis. They do not remain within the circulation. Clearance is diphasic with a significant drop in circulating lyophilized platelets within 10 minutes and majority clearance (60-70%) within 2 hours.[94] The remaining 30-40% demonstrate a half-life of around 24 hrs.[97] This may well be of relevance when considering the efficacy endpoint, platelet count at the T1, and T24 timepoints set in the current study.

Table 7: Canine thrombocytopenia study comparative data - Bleeding score, Platelet count and Haematocrit (HCT) median (+/- SD) and mean (range) results reported dependant on results available

Study GOGGS[26] Davidow[20] Levine[62]	Baseline entrance DOGIBAT /18 or bleeding score /5 # /16 ^	Baseline Entrance Plt count x10^9/l	Baseline entrance Haematocrit %	24 hour DOGIBAT /18 or bleeding score /5 # /16 ^	24hr Platelet count x 10^9/l	24hr Haematocrit %
GOGGS cryopreserved n=38	5 (3-7)	8 (0.8-12)	36.4 +/- 10.7	5 (3-6)	9 (1-35)	30 +/-9.6
GOGGS lyophilized n=50	6 (4-6)	6 (0.3-14.5)	36 +/-10.9	4.5 (3.25-6)	9 (2.3-18)	30.1 +/-9.3
Davidow fresh platelet concentrate n=15	3.27# (1.24)	18.53 (16.69)	32.3 (13.5)	2.32 (1.73)	32.21 (39.79)	28.1 (13.3)
Davidow lyophilized N=22	2.98 # (1.31)	16.45 (12.61)	28.9 (11.7)	2.73 (1.63)	20.66 (35.73)	25.6 (9.9)
Levine experimental ITP n=5	1^(0-1)	14 (11-28)	45.7 (32.9-47.6)	2^ (0-4)	32 (23-36)	37.8 (31.5-48.7)
Levine naturally Occurring primary ITP n=5	3^ (1-8)	0.5 (0-16)	40.5 (21.1-58.7)	n/a	n/a	n/a
Levine secondary ITP (ehrlichia) n=1	1^	16	46.6	n/a	n/a	n/a
Levine Thrombocytopenia other cause n=4	3.5^ (2-10/)	26.5 (0-29)	20.7 (15.7-29.1)	n/a	n/a	n/a
Current study Test group (subgroup Interim results) n=9	5.6 (3-8)	7.6 (0-22)	34.1 (22- 57)	5.2 (2-8)	26 (0-233)	30.9 (20-60)
Current study control group (subgroup Interim results) n=6	4.3 (3-5)	3.6 (0-20)	39 (22-50)	4.3 (1-8)	9.6 (0-39)	37 (24-45)

When looking at medical management within the cases enrolled, this was based upon the individual clinician and hospital protocol for treatment of underlying disease. Within this UK based study of twenty thrombocytopenic patients, nineteen cases were presumed to be immune mediated, one case was pancytopenia and sepsis, but with a previous history of immune mediated disease. Eighteen cases received corticosteroid, of the remaining two cases who did not receive corticosteroid, one had a diagnosis of ITP but administration of NSAIDS by the referring veterinarian prevented the attending referral clinician administering steroids due to a concern of risk of gastrointestinal ulceration. This patient was euthanased due to suspected intracranial haemmorrhage (ICH) 28 hours post administration of trial solution (T=28) before steroids were started, and one patient was thought to have ITP but died between t0 and t1 before administration of steroid. Timing of initiation of steroid treatment in the eighteen cases receiving it was six patients received it prior to or at t0, five patients received steroid at t1 and seven patients received it between t1 and t24. Five patients received antibiotics, of these five patients, one had a diagnosis secondary ITP (*Ehrlichia*), one was a septic pancytopenia with a presenting temperature of 40.2, one was an ITP with coexisting renal failure, one was secondary ITP (mammary neoplasia) and one was pyrexic on presentation with a white cell count of $45x10^9$/l.

Limitations to the UK study include number of enrolments as well as variability in the aetiology of the underlying conditions in the enrolled patient set, representing a heterogenous patient population. There was also a limitation in one of the key endpoint parameters. DOGiBAT is an observational scoring system. DOGiBAT scoring is designed to broadly assess the whole canine body for bleeding. However, in thrombocytopenia, where bleeding can be so widespread, including within body cavities, the brain, the lungs and the viscera, subtle microscopic bleeding and bleeding change is not detectable by readily available and repeatable on-demand diagnostic tests and observations. For example, in this study investigating a product that is known to have highest activity within hours rather than days, gaining faecal samples, urine samples or analysing a bruise/ecchymotic/petechial haemorrhage an hour apart,

on a split 24 hour timeline can be logistically impossible as well as too crude when considering subtle changes. This has been raised within similar human literature on bleeding thrombocytopenics.[67,85] Diagnostic findings in a thrombocytopenic patient of body cavity effusion, where thoracocentesis or abdominocentesis to define the effusion type would be contraindicated also reduces the ability to accurately score critical patients, hence the available scoring system in this setting can be defined as a limitation. However, it remains the most objective and appropriate scoring system for thrombocytopenic canines available at this time.

A multicentre design to enrol an adequate number of patients within such tight criteria, also means that there are multiple clinical leads and multiple individual establishment approaches to treatment. Although the majority of enrolled patients had broadly matching diagnoses of ITP, an absolute defined consensus on medical management of these critically presenting bleeding thrombocytopenic patients is not available. This is also compounded by the heterogenic nature of the underlying disease process in each patient.

Clients' decisions on amount of treatment to pursue for their pet may also have influence in some cases, where intensity of diagnostics and interventions beyond those consented to in the basic study were not guided purely by need alone but also by the clients' available finances and wishes for their own pet.

Protocol for treating ITP remains a subject under scrutiny in both humans,[14,28,30] and canines.[17,57] In human medicine, although corticosteroids remain the first line of treatment, in refractory cases, current focus is on more recently available drugs, such as TPO mimetics, anti-CD 20 monoclonal antibodies and TXA.[43] Platelet transfusion remains a controversial topic in human ITP, but is recommended in ITP cases with severe and life threatening haemorrhage.[14] Dosing protocols remain under discussion alongside the potential use of concurrent treatments such as combination infusion alongside IVIG. Despite ITP being the most common aetiology within this current canine study, the scope of the study did not include the

ability to explore or control treatment protocols, comprehensively designate the pathogeneses of the underlying disease processes and even in some cases ascertain definitive diagnoses.

So far at this interim stage, platelet transfusions as a safe option in bleeding thrombocytopenic canines appears to be supported in the most acute presentation to help in managing severe haemorrhage. This would of course always be alongside the institution of suitable medical management.[23]

Conclusion

This study at its current level of enrolment has not identified statistically significant efficacy claims between the two groups studied. There are no concerns for safety based on monitoring of adverse events.

One conclusion that can be drawn after feedback from clinical leads in the field is that lyophilized platelets are a realistic solution to a potential need. Bleeding thrombocytopenic canines are critical patients with a high risk of mortality due to bleeding complications.

Compared to the limited UK platelet transfusion options available, lyophilized platelets combine many practical benefits, the main one of immediate availability from the pharmacy shelf, combined with long shelf-stability and considerable ease of use compared to any other transfusion product available.

So far in preclinical and clinical studies, no additional risk to the recipient have been identified and significant adverse effect associated with administration of the product appears to be absent at this time.

The current aim is to continue to enrol to the proposed study number of 40. The study is being supported on an ongoing basis to look to achieve this level of enrolment and adequate test and control solution is available to do so.

Future research direction

In the pre-clinical human experimental setting, where a canine model of platelet refractoriness has been demonstrated after the administration of fresh platelet concentrate, it would be interesting to see how trehalose buffered lyophilized platelets perform with reference to the development of platelet refractoriness after repeated administration. It would also be interesting to see if in previously sensitized refractory patients they have the ability to act as a haemostatic bridge to manage these patients at times of acute crisis.

The ability to pre-load platelet concentrate prior to lyophilisation with additional therapeutic agents, examples such as vincristine and TXA, has been proven in the preclinical model, and for specific disease processes, the evolution of these combined therapies with the ability of the lyophilized platelet to target sites of bleeding are an interesting consideration.

In the veterinary sector, this product is now available for clinical use across the US for canine patients. Studies into its use in defined canine disease aetiology such as thrombocytopenia secondary to chemotherapy or neoplasia, the surgical management of thrombocytopenic animals, as well as in acute trauma resuscitation and critical bleeding (traumatic or surgical) may be of interest.

The consideration of bespoke dosing strategies based on disease entity alongside combination with other potentially synergistic or longer acting therapies according to disease aetiology may be an interesting direction. Exploring ways of more accurately monitoring individual patient progression and response to treatment, may also be helpful. Repeated dosing or constant rate infusion administration to control bleeding symptoms and stabilize haematocrit in critically bleeding patients may be an interesting method of exploring the most effective strategy for using trehalose buffered lyophilized platelets.

Additional supplementary material

Table 7: Baseline entrance Data - Continuous Variables Test versus Control

Variable	Group	Total	Nmissing	Mean	SE Mean	StDev	Minimum	Q1	Median	Q3	Maximum
	Test or Control										
Age at enrol	Test	11	0	6.689	0.915	3.034	1.671	5.005	7	7.581	12.008
	Control	9	0	5.68	1.27	3.82	1	1.56	5.16	9.5	10.05
Patient Weight	Test	11	0	22.23	2.77	9.2	9	12.6	22.3	28.7	37.8
	Control	9	0	13.86	2.11	6.33	6.5	9.6	11.3	19.5	25.6
Temperature	Test	10	1	38.87	0.22	0.695	37.7	38.175	39.1	39.4	39.9
	Control	9	0	38.289	0.305	0.914	36.9	37.75	38.2	38.75	40.2
HR	Test	10	1	114.4	6.03	19.06	80	97	120	126	140
	Control	9	0	104	7.78	23.35	80	84	96	120	150
RR	Test	10	1	33.4	2.57	8.11	24	28	30	42.5	48
	Control	9	0	36	8.69	26.06	20	22	30	34	104
DGB	Test	11	0	6	0.674	2.236	3	4	7	7	10
	Control	9	0	4.778	0.572	1.716	3	4	4	5	9
Platelet	Test	11	0	6.91	2.64	8.76	0	0	4	17	22
	Control	9	0	8.5	4.84	14.53	0	0	2	14.5	43

HCT	Test	11	0	0.352	0.029	0.0962	0.209	0.33	0.349	0.38	0.57
	Control	9	0	0.361	0.0321	0.0962	0.22	0.2855	0.349	0.4515	0.5
PT	Test	11	0	14.183	0.845	2.804	6.31	14	14.6	15.8	16.9
	Control	9	0	14.022	0.827	2.481	8.8	12.8	14.6	15.75	17
APTT	Test	11	0	95.66	9.17	30.42	7.73	97	102	112.9	117.6
	Control	9	0	106.2	3.25	9.75	88.9	98.8	107	114	120.2
wbc	Test	11	0	21.37	3.49	11.59	10.2	11	20.2	25.72	48
	Control	9	0	12.54	3.94	11.82	0.3	4.2	9.09	16.46	39.9
neut	Test	10	1	15.51	2.69	8.49	6.13	9.59	12.68	19.99	34.57
	Control	8	1	9.77	3.08	8.72	0	2.69	9.1	12.56	27.9
urea	Test	11	0	6.173	0.971	3.222	3.2	3.8	5.3	8.4	13.4
	Control	9	0	9.01	2.48	7.43	2.6	3.85	7.8	10.9	26.9
tbil	Test	9	2	38.4	31.8	95.5	3.8	6.5	7	7.5	293
	Control	9	0	4.667	0.833	2.5	3	3	4	5	11
Alb	Test	11	0	27.75	1.25	4.14	19	25	28	32	33
	Control	9	0	28.78	2.11	6.34	20	24	25	35.5	36
Days in Hospital	Test	8	3	4.375	0.706	1.996	1	3	4.5	6	7
	Control	7	2	4.571	0.922	2.44	2	2	4	7	8

Appendices

Appendix 1: Trehalose buffered lyophilized Platelets, Preclinical trial summary

PRECLINICAL TRIALS

Pre Clinical - Safety and efficacy trials

Safety trials into trehalose buffered lyophilized platelets prior to phase 2/3 clinical trials in both humans and canines, included an escalating dose study in canines using same species lyophilized platelets and including a comparison to the human current standard of care in platelet transfusion, fresh platelets (same species). In addition a cross species study was performed with human lyophilized platelet (Thrombosomes) looking at vessel bleeding times in the rabbit model, a maximum tolerated dose study in the rabbit model, a non human primate (NHP) model of blood loss and circulatory shock due to liver injury and a canine model of Coronary Artery bypass graft (CABG) comparing low, medium and high dose lyophilized platelets to fresh platelets and placebo (saline).

The safety data shown in Table 1 displays data from the highest dose level in the safety studies into trehalose buffered lyophilized platelets, based on particle count and potential thrombin generation and the potential peak thrombin (PT) and endogenous thrombin potential (ETP) associated with that dose level per µl of whole blood.

Additional data on all dose levels in each study are provided in the tables below. There have been no test article related safety adverse events in any nonclinical study at any dose level.

Table 1 – Highest Dose Level, Based on Particle Count and Potential Thrombin Generation, from Each Completed Study

Study Description	Total Particle Count Infused	Particles per KG	Particles per µL	Potential Thrombin Generation nM per µL	
				PT	ETP
Single High Dose Toxicity of Canine Thrombosomes in Dogs (Lyophilized Canine Platelets, LCP)	$7.54 \times 10^{10} \pm 3.7 \times 10^{9}$	$1.00 \times 10^{10} \pm 2.79 \times 10^{5}$	$1.18 \times 10^{5} \pm 3.29 \times 10^{0}$	$1.36 \times 10^{6} \pm 4.43 \times 10^{1}$	$1.29 \times 10^{8} \pm 3.68 \times 10^{3}$
Single High Dose Canine CABG Safety Study in Dogs (Lyophilized Canine Platelets, LCP)	$1.46 \times 10^{11} \pm 2.94 \times 10^{10}$	$5.11 \times 10^{9} \pm 2.88 \times 10^{8}$	$7.29 \times 10^{4} \pm 4.11 \times 10^{3}$	$8.40 \times 10^{5} \pm 4.73 \times 10^{4}$	$7.99 \times 10^{7} \pm 4.50 \times 10^{6}$

Preclinical: Efficacy Data

Pre-clinical studies where the efficacy of StablePlate RX® (LCP) was evaluated by measuring the effect on bleeding are displayed in Table 2.

These trials assessed the lowest dose levels demonstrating efficacy based on particle concentration and the associated PT and ETP with that concentration of particles.

Table 2 – **Lowest** Dose Level Demonstrating Efficacy, Based on Particle Count and Potential Thrombin Generation, from Each Completed Study that Measured the Effect on Bleeding

Study Description	Total Particle Count Infused	Particles per KG	Particles per µL	Potential Thrombin Generation nM per µL	
				PT	ETP
Single High Dose Canine CABG Safety Study in Dogs (Lyophilized Canine Platelets, LCP)	$4.09 \times 10^{10} \pm 7.23 \times 10^{9}$	$1.57 \times 10^{9} \pm 1.06 \times 10^{8}$	$2.24 \times 10^{4} \pm 1.51 \times 10^{3}$	$2.58 \times 10^{5} \pm 1.74 \times 10^{4}$	$2.45 \times 10^{7} \pm {}^{1.66}\ 10^{6}$

Preclinical Toxicity – cross species

The study summarized in table 3 is an acute single dose GLP safety study designed to determine a maximum tolerated dose (MTD) and was conducted in healthy New Zealand White Rabbits using thrombosomes (human lyophilized platelets). The maximum acute dose allowed by the CRO IACUC was 70mL of test article. The resulting dose is presented as a mass calculation particle per KG, particles per µL of blood volume and as a potency measure of potential nM of thrombin generated by the maximum concentration of lyophilized platelets per µL of blood volume. A MTD was not achieved as no adverse events were noted. A no observable adverse event level (NOAEL) was established based on mass of test article delivered.

Table 3 - Group 7 Thrombosomes®, human lyophilized platelets, N= 12 (6-M, 6-F), Volume administered 70ml (mean particle count 1.42 X 10^9 /ml ± 3.87 X 10^8)

Weight (KG)	Blood Volume (ml)	Volume of Test Article Infused	Total Particle Count Infused	Particles per KG	Particles per µL	Potential Thrombin[1] Generation nM per µL	
						PT	ETP
3.5 ± 0.28	196.09 ± 15.92	70.0ml	9.87 X 10^{10}	2.84 X 10^{10} ± 2.31 X 10^9	5.06 X 10^5 ± 4.13 X 10^4	5.83 X 10^6 ± 4.75 X 10^5	5.55 X 10^8 ± 4.52 X 10^7

Pre clinical canine same species safety study :

The study summarized in table 4, examined the safety of a single escalating dose of lyophilized canine platelets (LCP) and multiple doses, three doses of LCP 2h apart, followed 2h later by a dose of fresh canine platelets.

Table 4 - Single High Dose Toxicity of Canine LCP in Dogs (Lyophilized Canine Platelets, LCP)

Group	Weight (KG)	Blood Volume (ml)	Volume of Test Article Infused	Total Particle Count (LCP) Infused	Particles per KG	Particles per µL	Potential Thrombin Generation nM per µL	
							PT	ETP
Low Dose	7.76 ± 0.59	659.36 ± 49.9	69.3 ± 60.7	$1.33 \times 10^{10} \pm 3.94 \times 10^{9}$	$1.71 \times 10^{9} \pm 4.88 \times 10^{8}$	$2.02 \times 10^{4} \pm 5.74 \times 10^{3}$	$2.32 \times 10^{5} \pm 6.82 \times 10^{4}$	$2.21 \times 10^{7} \pm 6.29 \times 10^{6}$
Medium Dose	7.61 ± 0.44	647.06 ± 37.27	86.14 ± 4.96	$4.22 \times 10^{10} \pm 2.43 \times 10^{9}$	$5.55 \times 10^{9} \pm 9.52 \times 10^{4}$	$6.53 \times 10^{4} \pm 1.12 \times 10^{0}$	$7.52 \times 10^{5} \pm 1.93 \times 10^{1}$	$7.15 \times 10^{7} \pm 1.30 \times 10^{3}$
High Dose	7.52 ± 0.37	638.92 ± 31.33	100.99 ± 4.95	$7.54 \times 10^{10} \pm 3.7 \times 10^{9}$	$1.00 \times 10^{10} \pm 2.79 \times 10^{5}$	$1.18 \times 10^{5} \pm 3.29 \times 10^{0}$	$1.36 \times 10^{6} \pm 4.43 \times 10^{1}$	$1.29 \times 10^{8} \pm 3.68 \times 10^{3}$
Multiple Dose	7.48 ± 0.36	636.08 ± 32.33						
Dose 1			20.32 ± 1.03	$1.21 \times 10^{10} \pm 6.14 \times 10^{8}$	$1.61 \times 10^{9} \pm 2.13 \times 10^{5}$	$1.9 \times 10^{4} \pm 2.5 \times 10^{0}$	$2.19 \times 10^{5} \pm 3.52 \times 10^{1}$	$2.08 \times 10^{7} \pm 2.82 \times 10^{3}$
Dose 2			12.87 ± 0.66	$1.67 \times 10^{9} \pm 8.54 \times 10^{7}$	$2.23 \times 10^{8} \pm 6.05 \times 10^{4}$	$2.63 \times 10^{3} \pm 7.11 \times 10^{1}$	$3.03 \times 10^{4} \pm 1.46 \times 10^{1}$	$2.88 \times 10^{6} \pm 8.55 \times 10^{2}$
Dose 3			10.05 ± 0.51	$7.5 \times 10^{9} \pm 3.8 \times 10^{8}$	$1.00 \times 10^{9} \pm 2.56 \times 10^{5}$	$1.18 \times 10^{4} \pm 3.01 \times 10^{0}$	$1.36 \times 10^{5} \pm 4.10 \times 10^{1}$	$1.29 \times 10^{7} \pm 3.37 \times 10^{3}$

Preclinical side effect and efficacy study into Canine on pump coronary artery bypass graft (CABG) model

The study summarized in Table 5 was a single dose GLP safety study performed in a canine on-pump coronary artery bypass graft (CABG) model. Safety, was assessed through the collection of blood loss, evaluation of blood flow through the bypass graft, evaluation of the development of acute thrombosis, and maintenance of patency through the graft over a 4hr evaluation period. Dose levels of 3.3%, 10%, and 33% of the total circulating platelet count (TCP) were used as recommended by FDA. Doses of up to 33% of the animal's total circulating platelet count and associated thrombin generation potential were not associated with any unexpected mortality, adverse changes in hematology or coagulation parameters, development of thrombosis at the anastomosis sites. The results of this study also demonstrated that administration of the test article at doses of 10% and 33% of the TCP aided in the control of blood loss. These studies were conducted after the change in determining particle count and size distribution was validated and adopted, no adjustments to dose based on count were necessary.

Table 5 - Single High Dose Canine CABG Safety Study in Dogs (Lyophilized Canine Platelets, LCP)

Group	Weight (KG)	Blood Volume (ml)	Volume of Test Article Infused	Total Particle Count Infused	Particles per KG	Particles per µL	Potential Thrombin Generation nM per µL	
							PT	ETP
Dose 1 3.3% TCP	25.94 ± 3.47	1815.63 ± 243.03	8.21 ± 0.72	$1.36 \times 10^{10} \pm 1.68 \times 10^{9}$	$5.25 \times 10^{8} \pm 5.01 \times 10^{7}$	$7.50 \times 10^{3} \pm 7.15 \times 10^{2}$	$8.64 \times 10^{4} \pm 8.25 \times 10^{3}$	$8.22 \times 10^{6} \pm 7.84 \times 10^{5}$
Dose 2 10% TCP	26.08 ± 4.37	1825.25 ± 306.12	24.53 ± 4.58	$4.09 \times 10^{10} \pm 7.23 \times 10^{9}$	$1.57 \times 10^{9} \pm 1.06 \times 10^{8}$	$2.24 \times 10^{4} \pm 1.51 \times 10^{3}$	$2.58 \times 10^{5} \pm 1.74 \times 10^{4}$	$2.45 \times 10^{7} \pm 1.66 \times 10^{6}$
Dose 3 33% TCP	28.42 ± 4.7	1989.31 ± 328.86	89.19 ± 22.71	$1.46 \times 10^{11} \pm 2.94 \times 10^{10}$	$5.11 \times 10^{9} \pm 2.88 \times 10^{8}$	$7.29 \times 10^{4} \pm 4.11 \times 10^{3}$	$8.40 \times 10^{5} \pm 4.73 \times 10^{4}$	$7.99 \times 10^{7} \pm 4.50 \times 10^{6}$

Minimum effective dose (MED) discussion

The hemostatic effect of lyophilized platelets was investigated in three studies. A comparison of particle count and potential thrombin generation of the lowest dose having a hemostatic effect were presented in Table 2 above.

Blood loss data in a thrombocytopenic ear bleed rabbit model indicated efficacy when Thrombosomes (lyophilized human platelets) equal to 1% of the animal's total circulating platelet count (TCP) and their associated thrombin generation potential were efficacious, no lower dose was administered.

Blood loss data was also collected in a Non Human Primate (NHP) model of liver injury with circulatory shock, a trend indicating efficacy was observed at both dose levels but no dose dependence was apparent.

Blood loss data indicates that infusion of lyophilized canine particles equaling 10% of the animal's TCP and their associated thrombin generation potential were efficacious while infusions equaling 3.3% of the TCP were not efficacious in the canine CABG model.

Single High Dose Canine CABG Safety Study in Dogs (Lyophilized Canine Platelets, LCP)

A MED was observed in this study. Animals receiving doses based on 33% and 10% of the total circulating platelet count had significantly less bleeding than untreated animals and were comparable to animals treated with 2 day old room temperature stored canine platelets. Animals receiving doses based on 3.3% of the total circulating platelet count had blood loss similar to untreated animals and greater than to animals treated with 2 day old room temperature stored canine platelets. Indicating a MED based on 10% of the TCP, additional dosing studies would be needed to determine if doses less than 10% of the TCP would be effective (Figure 1).

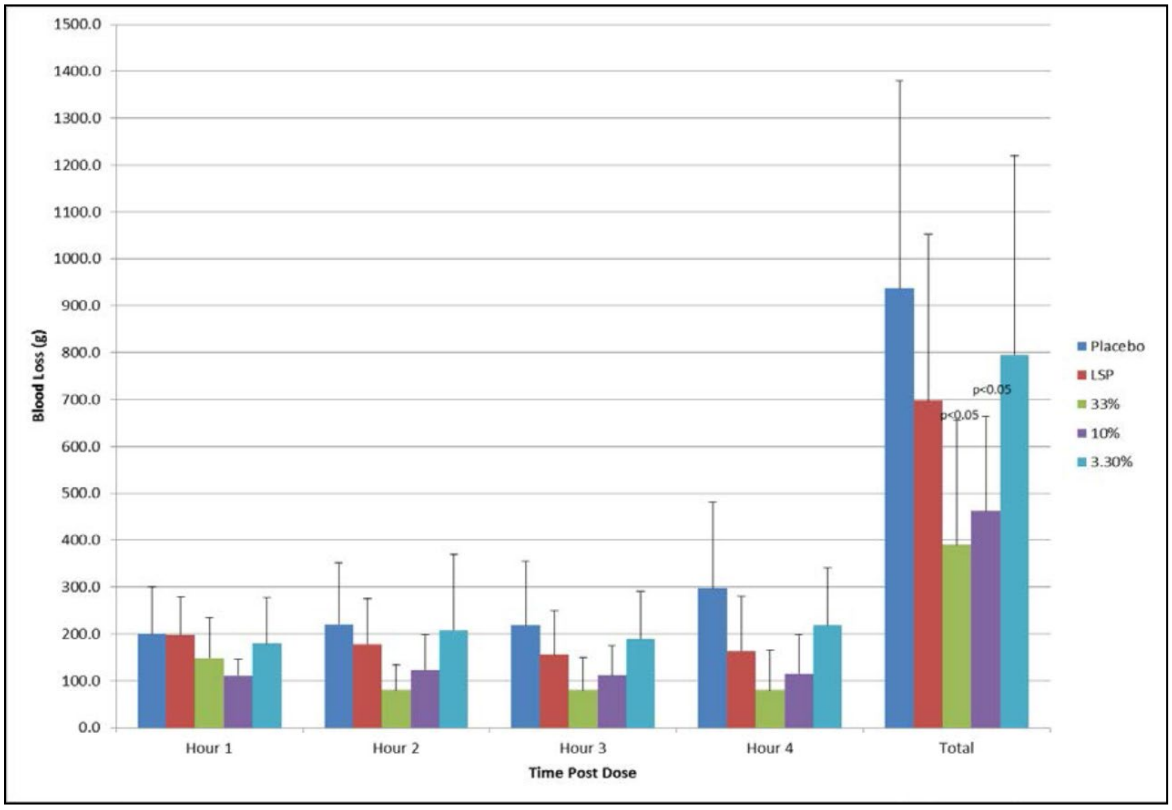


Figure 1. Comparison of hourly and total blood loss over a 4 hour period after completion of coronary artery bypass graft surgery in canine. Animals received saline (placebo), Two day old liquid stored platelets (LSP, 10% of the total circulating platelet count, TCP), or lyophilized canine platelets (LCP) at doses of 3.3, 10, 0r 33% of the animal's TCP. Animals receiving doses of 10% or 33% TCP had significantly decreased blood loss.

Conclusions of Preclincial work

These preclinical studies reflect the depth of investigation performed on the lyophilized canine platelet product, StablePlate RX®, prior to its release in the United States. Dosing structures that were 10X the current high end clinical dose (3.0×10^9 particles/kg) for severe hemorrhage were evaluated during the safety phase of this product development without related adverse reaction or event.

In addition, efficacy in a cardiopulmonary bypass model with arterial graft suggests an area of use in this situation within this area of clinical practice.

Appendix 2 : ATC certificate

Veterinary Medicines Directorate

Veterinary Medicines Directorate
Woodham Lane, New Haw
Addlestone, Surrey
KT15 3LS
United Kingdom

Tel: +44 (0)1932 336911
Search for VMD on GOV.UK

THE VETERINARY MEDICINES REGULATIONS 2013 SI 2013/2033

ANIMAL TEST CERTIFICATE No **ATC** 51028/4000
In the name of StablePlate RX®

Granted to: BodeVet® Inc
9210 Corporate Blvd Ste 310
Rockville
MD 20850
USA

In accordance with the Veterinary Medicines Regulations I approve the administration of **StablePlate RX®** to animals in accordance with the protocol submitted to the Veterinary Medicines Directorate on 06 March 2019.

This Certificate is valid for two years.

Application No: 01807/2018

Signature:

A person authorised to sign on behalf of the Secretary of State for Environment, Food and Rural Affairs.

Date: 08 May 2019

Appendix 3: Welfare and Ethics Certificate University of Edinburgh

18th June 2019

Veterinary Ethical Review Committee
The Royal (Dick) School of Veterinary Studies
The University of Edinburgh
Easter Bush Veterinary Centre
Roslin, Midlothian
EH25 9RG

MSc student Dr Jenny Walton
Supervisors Professor Richard Mellanby
Dr Simon Tappin
R(D)SVS, HFSA, The University of Edinburgh
Company: BodeVet, Inc.
9430 Key West Avenue Ste 250 Rockville,
MD 20850 USA

Dear Dr Walton, Prof Mellanby & Dr Tappin,

I can confirm that the project "Evaluation of StablePlate RX® in Thrombocytopenic canine patients: a multicentre clinical trial" has been reviewed by the R(D)SVS Veterinary Ethical Review Committee and received ethical approval.

This VERC approval comes with the following conditions (as specified in the proposal):

- Any severe adverse events that halts the study should be reported to VERC
- VERC should review the interim report before a second phase of the study is approved

The VERC reference number is 78.19.

We wish you every success with your trial.

Yours sincerely,

Dr Gurå Therese Bergkvist
FHEA, BSc (Hons), BVM&S, PhD, MRCVS

Chair, R(D)SVS Veterinary Ethical Review Committee

Appendix 4: Welfare and Ethics Certificate Royal Veterinary College – London

Royal Veterinary College
University of London

Royal Veterinary College
Royal College Street
London
NW1 0TU
DDL +44(0)20 7468 5300
Fax +44(0)20 7388 1027
E lwilkinson@rvc.ac.uk
www.rvc.ac.uk

Karen Humm
CSS
Hawkshead

10 May 2019

Dear Karen

URN 2019 1880-3 - Project Title: Evaluation of StablePlate RX ® in thrombocytopaenic canine patients: a multicentre clinical trial
Anticipated Start: 2019 May - Duration: 1 year
Location: The RVC part of the study is at the QMHA, but this is part of a multi-centre study including the RDSVS small animal hospital – University of Edinburgh, Vets Now Glasgow, Dick White Referrals and Davies Veterinary Specialists

I am pleased to advise that this project has been ethically reviewed by the Clinical Research Ethical Review Board (CRERB) and that ethical approval has been granted. Please ensure that you put your reference (URN) number on any documentation relating to the study and indicate that ethics approval has been given by the Clinical Research and Ethical Review Board at the Royal Veterinary College. You also need to keep a copy of this letter in the study file.

If during your project, you come across any ethical concerns then it is important that you let the Committee know so that they are aware and provide advice if applicable. At the end of your project we will be in contact to ask if there were any ethical issues that arose and you might be asked to complete a formal report.

It is important that we are kept informed of when this project has finished. If your project finishes early please let me know. Likewise if your project goes beyond the scheduled finish date you need to contact me to ask for an extension.

We hope that your project goes well. If you need to make any changes to the project, then please contact me for an amendment form.

Yours sincerely

Liz Wilkinson
Secretary: Clinical Research Ethical Review Board

Cc: Tracy Van Der Merwe; Barbora King (CIC/QMH)

Appendix 5: Clinical intro PowerPoint

See supplementary files – PowerPoint

Appendix 6: Detailed Clinical Protocol

See supplementary files – PDF 1-2019-K9 UK Detailed Clinical Protocol

Appendix 7: Investigators Brochure

See supplementary files – PDF 1-2019-K9 UK Investigators Brochure

Bibliography

1. Hitchcock IS, Kaushansky K. Thrombopoietin from beginning to end. British Journal of haematology 2014;165:259-268 doi:10.1111/bjh.12772

2. Patel SR, Hartwig JH. The Biogenesis of platelets from megaaryocyte proplatelets. Journal of clinical invetigation2005;115(12):3348-3354. doi:10.1172/JCI26891.3348

3. Boudreaux MK, Ebbe S. Comparison of platelet number, mean platelet volume and platelet mass in five mammalian species. *Comp Haematol Int*. 1998;8(1):16-20. doi:10.1007/BF02628099

4. Noris P, Klersy C, Gresele P, et al. Platelet size for distinguishing between inherited thrombocytopenias and immune thrombocytopenia: A multicentric, real life study. *Br J Haematol*. 2013;162(1):112-119. doi:10.1111/bjh.12349

5. Cortese L, Christopherson PW, Pelagalli A. Platelet function and therapeutic applications in dogs: Current status and future prospects. *Animals*. 2020;10(2):1-26. doi:10.3390/ani10020201

6. Reddy EC, Rand ML. Procoagulant Phosphatidylserine-Exposing Platelets in vitro and in vivo. *Front Cardiovasc Med*. 2020;7(March):1-11. doi:10.3389/fcvm.2020.00015

7. Dachary-Prigent J, Freyssinet JM, Pasquet JM, Carron JC, Nurden AT. Annexin V as a probe of aminophospholipid exposure and platelet membrane vesiculation: A flow cytometry study showing a role for free sulfhydryl groups. *Blood*. 1993;81(10):2554-2565. doi:10.1182/blood.v81.10.2554.2554

8. Schalm, O.W. et al., 2000. Schalm's Veterinary hematology Fifth edition., Baltimore, Md. LLW& W; 2000:443-466.

9. Cremer SE, Catalfamo JL, Goggs R, Seemann SE, Kristensen AT, Brooks MB. Proteomic profiling of the thrombin-activated canine platelet secretome (CAPS). *PLoS One*. 2019;14(11):1-22. doi:10.1371/journal.pone.0224891

10. Smith SA. The cell-based model of coagulation: State-Of-The-Art Review. *J Vet Emerg Crit Care*. 2009;19(1):3-10. doi:10.1111/j.1476-4431.2009.00389.x

11. Brass LF. Thrombin and platelet activation Chest journal 2003 (3);124:18s-25s

12. Cowan SM, Bartges JW, Gompf RE, et al. Giant platelet disorder in the Cavalier King Charles Spaniel. *Exp Hematol*. 2004;32(4):344-350. doi:10.1016/j.exphem.2004.01.008

13. Hayakawa S, Spangler EA, Christopherson PW, Boudreaux MK. A novel form of macrothrombocytopenia in Akita dogs. Vet Clin Pathol. 2016;45:103–105

.14. Estcourt LJ, Birchall J, Allard S, et al. Guidelines for the use of platelet transfusions. *Br J Haematol*. 2017;176(3). doi:10.1111/bjh.14423

15. Grindem CB1, Breitschwerdt EB, Corbett WT JH. Epidemiologic survey of thrombocytopenia in dogs: a report on 987 casesle. *Vet Clin Pathol*. 1991;20:38-43. doi:10.1111/j.1939-165x.1991.tb00566.x

16. Botsch V, Küchenhoff H, Hartmann K, Hirschberger J. Retrospective study of 871 dogs with thrombocytopenia. *Vet Rec*. 2009;164(21):647-651. doi:10.1136/vr.164.21.647

17. Callan MB, Appleman EH, Sachais BS. Canine platelet transfusions: State-of-the-Art Review. *J Vet Emerg Crit Care*. 2009;19(5):401-415. doi:10.1111/j.1476-4431.2009.00454.x

18. Hux BD, Martin LG. Platelet transfusions: Treatment options for hemorrhage secondary to thrombocytopenia. *J Vet Emerg Crit Care*. 2012;22(1):73-80. doi:10.1111/j.1476-4431.2011.00706.x

19. Ng ZY, Stokes JE, Alvarez L, Bartges JW. Cryopreserved platelet concentrate transfusions in 43 dogs: a retrospective study (2007–2013). *J Vet Emerg Crit Care*. 2016;26(5):720-728. doi:10.1111/vec.12503

20. Davidow EB, Brainard B, Martin LG, et al. Use of fresh platelet concentrate or lyophilized platelets in thrombocytopenic dogs with clinical signs of hemorrhage: A preliminary trial in 37 dogs. *J Vet Emerg Crit Care*. 2012;22(1):116-125. doi:10.1111/j.1476-4431.2011.00710.x

21. https://www.icd10data.com/ICD10CM/Index/T/Thrombocytopenia%2c_thrombocytopenic.

22. Cooper SA, Huang AA, Raskin RE, Weng HY, Scott-Moncrieff JC. Clinical data, clinicopathologic findings and outcome in dogs with amegakaryocytic thrombocytopenia and primary immune-mediated thrombocytopenia. *J Small Anim Pract*. 2016;57(3):142-147. doi:10.1111/jsap.12441

23. Putsche JC, Kohn B. Primary immune-mediated thrombocytopenia in 30 dogs (1997-2003). *J Am Anim Hosp Assoc*. 2008;44(5):250-257. doi:10.5326/0440250

24. Bianco D, Armstrong PJ, Washabau RJ. Treatment of severe immune-mediated thrombocytopenia with human IV immunoglobulin in 5 dogs. *J Vet Intern Med*. 2007;21(4):694-699. doi:10.1892/0891-6640(2007)21[694:TOSITW]2.0.CO;2

25. Goggs R, Boag AK, Chan DL. Concurrent immune-mediated haemolytic anaemia and severe thrombocytopenia in 21 dogs. *Vet Rec*. 2008;163(11):323-327. doi:10.1136/vr.163.11.323

26. Goggs R, Brainard BM, LeVine DN, et al. Lyophilized platelets versus cryopreserved platelets for management of bleeding in thrombocytopenic dogs: A multicenter randomized clinical trial. *J Vet Intern Med*. 2020. doi:10.1111/jvim.15922

27. Bachman DE, Forman MA, Hostutler RA, Corn S, Lin J, Kociba GJ. Prospective diagnostic accuracy evaluation and clinical utilization of a modified assay for platelet-associated immunoglobulin in thrombocytopenic and nonthrombocytopenic dogs. *Vet Clin Pathol*. 2015;44(3):355-368. doi:10.1111/vcp.12281

28. Galdarossa M, Vianello F, Tezza F, et al. Epidemiology of primary and secondary thrombocytopenia: First analysis of an administrative database in a major Italian institution. *Blood Coagul Fibrinolysis*. 2012;23(4):271-277. doi:10.1097/MBC.0b013e328351882d

29. Neylon AJ, Saunders PWG, Howard MR, Proctor SJ, Taylor PRA. Clinically significant newly presenting autoimmune thrombocytopenic purpura in adults: A prospective study of a population-based cohort of 245 patients. *Br J Haematol*. 2003;122(6):966-974. doi:10.1046/j.1365-2141.2003.04547.x

30. Humbrecht C, Kientz D, Gachet C. Platelet transfusion: Current challenges. *Transfus Clin Biol*.

2018;25(3):151-164. doi:10.1016/j.tracli.2018.06.004

31. Slichter SJ, Kaufman RM, Assmann SF, et al. Dose of prophylactic platelet transfusions and prevention of hemorrhage. *N Engl J Med*. 2010;362(7):600-613. doi:10.1056/NEJMoa0904084

32. Slichter SJ. Relationship between platelet count and bleeding risk in thrombocytopenic patients. *Transfus Med Rev*. 2004;18(3):153-167. doi:10.1016/j.tmrv.2004.03.003

33. Rodeghiero F, Stasi R, Gernsheimer T, et al. Standardization of terminology , definitions and outcome criteria in immune thrombocytopenic purpura of adults and children : report from an international working group. 2009;113(11):2386-2393. doi:10.1182/blood-2008-07-162503.

34. Kistangari G, McCrae KR. Immune thrombocytopenia. *Hematol Oncol Clin North Am*. 2013;27(3):495-520. doi:10.1016/j.hoc.2013.03.001

35. Rodeghiero F. Is ITP a thrombophilic disorder? *Am J Hematol*. 2016;91(1):39-45. doi:10.1002/ajh.24234

36. Itp TP, Negi RR, Bhoria P, et al. Investigation of the Possible Association between the HLA Antigens and Idiopathic Thrombocytopenic Purpura (ITP). 2012;0139. doi:10.3109/08820139.2011.593218

37. Salama OS, Aladl DA, El Ghannam DM, Elderiny WE. Evaluation of platelet cross-matching in the management of patients refractory to platelet transfusions. *Blood Transfus*. 2014;12(2):187-194. doi:10.2450/2014.0120-13

38. Olsson B, Andersson P, Jernås M, et al. T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. 2003;9(9):1123-1124. doi:10.1038/nm921

39. Provan D et al Updated international consensus report on the investigation and management of primary immune thrombocytopenia. *Blood Adv* 2019; 3 (22): 3780–3817. doi: https://doi.org/10.1182/bloodadvances.2019000812

40. Psaila B, Bussel JB. Refractory immune thrombocytopenic purpura: Current strategies for investigation and management. *Br J Haematol*. 2008;143(1):16-26. doi:10.1111/j.1365-2141.2008.07275.x

41. Yan Z, Xin D, Jiajun X, Xun N. TPO receptor agonist for chronic idiopathic thrombocytopenic purpura. *Cochrane Database Syst Rev*. 2011;(7):2-4. doi:10.1002/14651858.CD008235.pub2.Copyright

42. Nakamura RK, Tompkins E, Bianco D. Therapeutic options for immune-mediated thrombocytopenia. *J Vet Emerg Crit Care*. 2012;22(1):59-72. doi:10.1111/j.1476-4431.2011.00705.x

43. Estcourt LJ, McQuilten Z, Powter G, et al. The TREATT Trial (TRial to EvaluAte Tranexamic acid therapy in Thrombocytopenia): Safety and efficacy of tranexamic acid in patients with haematological malignancies with severe thrombocytopenia: Study protocol for a double-blind randomised controlled tri. *Trials*. 2019;20(1):1-14. doi:10.1186/s13063-019-3663-2

44. Spahr, J.E. and Rodgers, G.M. (2008), Treatment of immune-mediated thrombocytopenia purpura with concurrent intravenous immunoglobulin and platelet transfusion: A retrospective review of 40 patients. Am. J. Hematol., 83: 122-125. https://doi.org/10.1002/ajh.21060J

45. Psaila B, Petrovic A, Page LK, Menell J, Schonholz M, Bussel JB. Intracranial hemorrhage (ICH) in children with immune thrombocytopenia (ITP): Study of 40 cases. *Blood*. 2009;114(23):4777-4783. doi:10.1182/blood-2009-04-215525

46. Heffner RR, Kahn EE, Schraft WC. Thrombotic thrombocytopenic purpura. *N Y State J Med*. 1954;54(1):111-113. doi:10.1182/blood-2016-10-709857.2836

47. Williams DA,Maggio Price L CAnine idiopathic thrombocytopenia clinical observations and long term follow up in 54 cases. Journal of American Veterinary Medical Association 1984 185(6):660-663.

48. Jackson ML, Kruth SA. Immune-mediated Hemolytic Anemia and Thrombocytopenia in the Dog : A retrospective study of 55 cases diagnosed from 1969 through 1983 at the Western College of Veterinary Medicine. 1985:245-250.

49. Cummings FO, Rizzo SA. Treatment of presumptive primary immune-mediated thrombocytopenia with mycophenolate mofetil versus cyclosporine in dogs. *J Small Anim Pract*. 2017;58(2):96-102. doi:10.1111/jsap.12621

50. Simpson K, Chapman P, Klag A. Long-term outcome of primary immune-mediated thrombocytopenia in dogs. *J Small Anim Pract*. 2018;59(11):674-680. doi:10.1111/jsap.12912

51. O'Marra SK, Delaforcade AM, Shaw SP. Treatment and predictors of outcome in dogs with immune-mediated thrombocytopenia. *J Am Vet Med Assoc*. 2011;238(3):346-352. doi:10.2460/javma.238.3.346

52. Rozanski EA, Callan MB, Hughes D, Sanders N, Giger U. Comparison of platelet count recovery with use of vincristine and prednisone or prednisone alone for treatment for severe immune-mediated thrombocytopenia in dogs. *J Am Vet Med Assoc*. 2002;220(4):477-481. doi:10.2460/javma.2002.220.477

53. Scuderi MA, Snead E, Mehain S, Waldner C, Epp T. Outcome based on treatment protocol in patients with primary canine immune-mediated thrombocytopenia: 46 cases (2000-2013). *Can Vet J*. 2016.

54. Kristensen AT, Weiss DJ, Klausner JS. Platelet Dysfunction Associated With Immune-Mediated Thrombocytopenia in Dogs. *J Vet Intern Med*. 1994.

55. Lewis DC, Meyers KM. Canine idiopathic thrombocytopenic purpura. *J Vet Intern Med*. 1996;10(4):207-218. doi:10.1111/j.1939-1676.1996.tb02052.x

56. Shropshire S, Dow S, Lappin M. Detection and dynamics of anti-platelet antibodies in thrombocytopenic dogs with and without idiopathic immune thrombocytopenia. 2020;(March 2019):700-709. doi:10.1111/jvim.15737

57. Abrams-Ogg ACG. Triggers for prophylactic use of platelet transfusions and optimal platelet dosing in thrombocytopenic dogs and cats. *Vet Clin North Am - Small Anim Pract*. 2003;33(6):1401-1418. doi:10.1016/S0195-5616(03)00095-0

58. Grindem CB, Breitschwerdt EB, Corbett WT, Page RL, Jans HE. Thrombocytopenia Associated With Neoplasia in Dogs. *J Vet Intern Med*. 1994;8(6):400-405. doi:10.1111/j.1939-1676.1994.tb03258.x

59. Balog K, Huang AA, Sum SO, Moore GE, Thompson C, Scott-Moncrieff JC. A prospective randomized clinical trial of vincristine versus human intravenous immunoglobulin for acute adjunctive management of presumptive primary immune-mediated thrombocytopenia in dogs. *J Vet Intern Med*. 2013.

60. Threlfall AJ, Boag AM, Soutter F, Glanemann B, Syme HM, Catchpole B. Analysis of DLA-DQB1 and polymorphisms in CTLA4 in Cocker spaniels affected with immune-mediated haemolytic anaemia. *Canine Genet Epidemiol*. 2020. doi:10.1186/s40575-015-0020-y

61. Stanworth SJ, Turner DM, Brown J, et al. Major Histocompatibility Complex Susceptibility Genes and Immune Thrombocytopenic Purpura in Caucasian Adults Major Histocompatibility Complex Susceptibility Genes and Immune Thrombocytopenic Purpura in Caucasian Adults. 2013;8454:4-7. doi:10.1080/10245330290028605

62. Levine DN, Adam J, Brooks MB, et al. A novel canine model of immune thrombocytopenia : has immune thrombocytopenia (ITP) gone to the dogs ? 2014;(July):110-120. doi:10.1111/bjh.13005

63. Michel M, Morin AS, Piette JC, et al. The spectrum of Evans syndrome in adults : new insight into the disease based on the analysis of 68 cases. *Blood*. 2009;114(15):3167-3173. doi:10.1182/blood-2009-04-215368.The

64. Makielski KM, Brooks MB, Wang C, Cullen JN, O'Connor AM, LeVine DN. Development and implementation of a novel immune thrombocytopenia bleeding score for dogs. *J Vet Intern Med*. 2018. doi:10.1111/jvim.15089

65. Page LK, Psaila B, Provan D, et al. The immune thrombocytopenic purpura (ITP) bleeding score: Assessment of bleeding in patients with ITP. *Br J Haematol*. 2007;138(2):245-248. doi:10.1111/j.1365-2141.2007.06635.x

66. Flores A, Buchanan GR. Occult Hemorrhage in Immune Thrombocytopenia. *Semin Hematol*. 2013;50(SUPPL.1):S26-S30. doi:10.1053/j.seminhematol.2013.03.006

67. Uhl L, Assmann SF, Hamza TH, Harrison RW, Gernsheimer T, Slichter SJ. Laboratory predictors of bleeding and the effect of platelet and RBC transfusions on bleeding outcomes in the PLADO trial. *Blood*. 2017;130(10):1247-1258. doi:10.1182/blood-2017-01-757930

68. Han T. Effect of Platelet Transfusion on Hemorraghe in patients with acute leukemia. *Cancer*. 1966;19:1937-1942.

69. Kaufman RM, Djulbegovic B, Gernsheimer T, et al. Platelet transfusion: A clinical practice guideline from the AABB. *Ann Intern Med*. 2015;162(3):205-213. doi:10.7326/M14-1589

70. World Health Organization. WHO Model List of Essential Medicines: 20th list. *WHO Med web http//www who int/medicines/publications/EML*. 2017;(March):62 p. doi:10.1016/S1473-3099(14)70780-7

71. Kral JB, Schrottmaier WC, Salzmann M, Assinger A. Platelet Interaction with Innate Immune Cells. *Transfus Med Hemotherapy*. 2016;43(2):78-88. doi:10.1159/000444807

72. Tinegate H, Birchall J, Gray A, et al. Guideline on the investigation and management of acute transfusion reactions Prepared by the BCSH Blood Transfusion Task Force. *Br J Haematol*. 2012;159(2):143-153. doi:10.1111/bjh.12017

73. National Blood Transfusion Committee Biennial Report (2017 / 19). 2019:1-20.

74. Estcourt LJ, Crighton GL, Wood EM, et al. A therapeutic-only versus prophylactic platelet transfusion strategy for preventing bleeding in patients with haematological disorders after chemotherapy or stem cell transplantation. *Cochrane Database Syst Rev*. 2014;2014(3). doi:10.1002/14651858.CD010981

75. https://www.transfusionguidelines.org/red-book/chapter-7-specifications-for-blood-components/7-10-platelets-apheresis-leucocyte-depleted.

76. Lyde RB, Poncz M, Lambert MP. Platelet transfusions. *Platelets Thromb Non-Thrombotic Disord Pathophysiol Pharmacol Ther an Updat*. 2017:1391-1401. doi:10.1007/978-3-319-47462-5_94

77. Squires JE. Indications for platelet transfusion in patients with thrombocytopenia. *Blood Transfus*. 2015;13(2):221-226. doi:10.2450/2014.0105-14

78. Heddle NM, Cook RJ, Tinmouth A, et al. A randomized controlled trial comparing standard- and low-dose strategies for transfusion of platelets (SToP) to patients with thrombocytopenia. *Blood*. 2009;113(7):1564-1573. doi:10.1182/blood-2008-09-178236

79. Frederiksen H, Christiansen CF, Norgaard M. Risk and prognosis of adult primary immune thrombocytopenia. *Expert Rev Hematol*. 2012;5(2):219-228. doi:10.1586/ehm.12.7

80. Desborough MJR, Smethurst PA, Estcourt LJ, Stanworth SJ. Alternatives to allogeneic platelet transfusion. *Br J Haematol*. 2016;175(3):381-392. doi:10.1111/bjh.14338

81. Corash L. Bacterial contamination of platelet components: Potential solutions to prevent transfusion-related sepsis. *Expert Rev Hematol*. 2011;4(5):509-525. doi:10.1586/ehm.11.53

82. Schrezenmeier H, Seifried E. Buffy-coat-derived pooled platelet concentrates and apheresis platelet concentrates: Which product type should be preferred? *Vox Sang*. 2010;99(1):1-15. doi:10.1111/j.1423-0410.2009.01295.x

83. Heddle NM, Arnold DM, Boye D, Webert KE, Resz I, Dumont LJ. Comparing the efficacy and safety of apheresis and whole blood-derived platelet transfusions: A systematic review. *Transfusion*. 2008;48(7):1447-1458. doi:10.1111/j.1537-2995.2008.01731.x

84. Gilliss BM, Looney MR, Gropper MA. Reducing Noninfectious Risks of Blood Transfusion. *Anesthesiology*. 2011;115(3):635-649. doi:10.1097/ALN.0b013e31822a22d9

85. Slichter SJ. controversies in platelet transfusion therapy ann,rev.med 1980.31;509-540

86. https://www.bsavalibrary.com/content/formulary/backmatter/canine-and-felinebodyweightbwtobodysurfaceareabsaconversiontables.

87. Bode AP, Fischer TH. Lyophilized platelets: Fifty years in the making. *Artif Cells, Blood Substitutes, Biotechnol*. 2007;35(1):125-133. doi:10.1080/10731190600974962

88. DiCarlo AL, Maher C, Hick JL, Hanfling D, Dainiak N, Chao N, Bader JL, Coleman CN, Weinstock DM. Radiation injury after a nuclear detonation: medical consequences and the need for scarce resources allocation. Disaster Med Public Health Prep. 2011 Mar;5 Suppl 1(0 1):S32-44. doi: 10.1001/dmp.2011.17. PMID: 21402810; PMCID: PMC3643117.

89. Cardenas JC, Zhang X, Fox EE, et al. Platelet transfusions improve hemostasis and survival in a

substudy of the prospective, randomized PROPPR trial. *Blood Adv*. 2018;2(14):1696-1704. doi:10.1182/bloodadvances.2018017699

90. Michael Fitzpatrick G. Novel Platelet Products under Development for the Treatment of Thrombocytopenia or Acute Hemorrhage. *Transfus Apher Sci*. 2018. doi:10.1016/j.transci.2018.12.010

91. Epstein JS. Best practices in regulation of blood and blood products. *Biologicals*. 2012;40(3):200-204. doi:10.1016/j.biologicals.2011.11.002

92. Bode AP, Lust RM, Read MS, Fischer TH. Correction of the bleeding time with lyophilized platelet infusions in dogs on cardiopulmonary bypass. *Clin Appl Thromb*. 2008;14(1):38-54. doi:10.1177/1076029607304746

93. Wolkers WF, Walker NJ, Tablin F, Crowe JH. Human platelets loaded with trehalose survive freeze-drying. *Cryobiology*. 2001;42(2):79-87. doi:10.1006/cryo.2001.2306

94. Fitzpatrick GM, Cliff R, Tandon N. Thrombosomes: A platelet-derived hemostatic agent for control of noncompressible hemorrhage. *Transfusion*. 2013;53(SUPPL. 1):100-106. doi:10.1111/trf.12043

95. Bode AP, Read MS, Reddick RL. Activation and adherence of lyophilized human platelets on canine vessel strips in the Baumgartner perfusion chamber. *J Lab Clin Med*. 1999;133(2):200-211. doi:10.1016/S0022-2143(99)90013-6

96. Wardrop KJ, Birkenheuer A, Blais MC, et al. Update on Canine and Feline Blood Donor Screening for Blood-Borne Pathogens. *J Vet Intern Med*. 2016;30(1). doi:10.1111/jvim.13823

97. Vibhudutta Awasthi, Josh Dee, Mike Fitzpatrick, Anna Koh, Richard Cliff GF. Trehalose Stabilized Freeze Dried Human Platelets, Thrombosomes®, Persist in Circulation 24 Hours After Infusion and Are Non-Immunogenic In New Zealand White Rabbits. https://www.bodevet.com/wp-content/uploads/2019/07/Trehalose-Stabilized-Freeze-Dried-Human-Platelets-Thrombosomes%C2%AE-Persist-in-Circulation-24-Hours-After-Infusion-and-Are-Non-Immunogenic-In-New-Zealand-White-Rabbits.pdf

98. Stanworth SJ, Navarrete C, Estcourt L, Marsh J. Platelet refractoriness - practical approaches and ongoing dilemmas in patient management. *Br J Haematol*. 2015;171(3):297-305. doi:10.1111/bjh.13597

99. Shehata N, Tinmouth A, Naglie G, Freedman J, Wilson K. ABO-identical versus nonidentical platelet transfusion: A systematic review. *Transfusion*. 2009;49(11):2442-2453. doi:10.1111/j.1537-2995.2009.02273.x

100. Curtis BR, Edwards JT, Hessner MJ, Klein JP, Aster RH. Blood group A and B antigens are strongly expressed on platelets of some individuals. *Blood*. 2000;96(4):1574-1581. doi:10.1182/blood.v96.4.1574.h8001574_1574_1581

101. Schrezenmeier H, Höchsmann B, Wiesneth M. How do we treat? Clinical haemotherapy: platelet transfusion. *ISBT Sci Ser*. 2010;5(n1):107-113. doi:10.1111/j.1751-2824.2010.01395.x

102. Slichter SJ, Abrams K, Gettinger I, et al. Ultraviolet B irradiation in the prevention of alloimmunization in a dog platelet transfusion model. *Transfusion*. 2016;56(6):1340-1347. doi:10.1111/trf.13516

103. Di Minno G, Perno CF, Tiede A, et al. Current concepts in the prevention of pathogen transmission via blood/plasma-derived products for bleeding disorders. *Blood Rev*. 2016;30(1):35-48. doi:10.1016/j.blre.2015.07.004

104. Birchall J, Haematologist C. Reactions to platelets suspended in additive solution or plasma : Data from SHOT. 2017;(September).

105. Oyama MA, Ellenberg SS, Shaw PA. Clinical Trials in Veterinary Medicine: A New Era Brings New Challenges. *J Vet Intern Med*. 2017;31(4):970-978. doi:10.1111/jvim.14744

106. Cancelas Perez JA, Ohanian M, Davenport R, Pullarkat V et al; 2019. Abstract PL2-MN4-32; A phase 1, open label, multicenter, dose escalation safety trial of lyophilized platelets in bleeding thrombocytopenic patients. AABB Annual Meeting;2019 Oct 19-22; San Antonio, TX.

107. Slichter SJ, Davis K, Enright H, et al. Factors affecting posttransfusion platelet increments , platelet refractoriness , and platelet transfusion intervals in thrombocytopenic patients. 2005;105(10):4106-4114. doi:10.1182/blood-2003-08-2724.