Platelet indices and their association with chronic valvular heart disease in Cavalier King Charles spaniels

Linda J Tong BVSc (Hons) MANZCVS

This thesis is presented for the degree of Research Masters with Training at Murdoch University, 2017

Declaration

I declare that this thesis is my own account of my research, and contains as its main content, work which has not previously been submitted for a degree at any tertiary education institution.

.....

Linda J Tong

Abstract

Chronic valvular heart disease (CVHD) is common in Cavalier King Charles spaniel (CKCS) and has the potential to affect platelet activation or function.

The present study objective was to determine platelet closure time, mean platelet component (MPC) concentration and platelet component distribution width (PCDW) in dogs with subclinical CVHD, and to assess the factors influencing these variables. A second objective was to assess platelet count, MPC concentration, PCDW, mean platelet volume (MPV), platelet volume distribution width (PDW), plateletcrit (PCT), mean platelet mass (MPM) and platelet mass distribution width (PMDW) in CKCS.

Haematological values, closure time, murmur grade and echocardiographic variables were recorded in 89 CKCS. Associations between explanatory variables (sex, age, murmur grade, echocardiographic variables, platelet count, and haematocrit (HCT)) and outcomes (closure time, MPC concentration, and PCDW) were examined using multivariate regression analysis. Platelet indices were compared between CKCS and a group of 39 control dogs (non-CKCS).

A model with 5 variables best explained variation in closure time (R^2, 0.74), with greater than 60% of the variance of closure time explained by mitral valve regurgitant jet size. The model of best fit to explain variation in MPC concentration included only platelet count (R^2, 0.24). The model of best fit to explain variation in PCDW included platelet count and sex (R^2, 0.25). The MPC concentration, MPV, PDW, MPM and PMDW values were significantly higher, and the platelet count, PCT, PCDW and HCT values significantly lower, in the CKCS compared to control dogs.

In the present study, a significant effect of mitral valve regurgitant jet size on closure time was consistent with platelet dysfunction. However, platelet activation, as determined by MPC concentration and PCDW, was not a feature of subclinical CVHD in CKCS.

Contents

Acknowledgements

I acknowledge with much gratitude, the support and mentorship provided by my principal supervisor, Dr Robert Shiel. I am indebted to Dr Shiel for his contribution to study conception and interpretation, the many discussions that were always productive, invaluable assistance with manuscript preparation and his unique and admirable ability to think outside the square.

I wish to express my thanks to my secondary supervisors, Professor Peter Irwin and Dr Susan Bennett; Professor Irwin for his valued guidance with thesis writing and timely encouragement, and Dr Bennett for her advice and assistance with cardiac auscultation on the clinic floor.

I acknowledge with thanks, Professor Hosgood for performing the statistical analysis and contribution to study design, which I feel has afforded this study a greater depth and dimension.

It is also with gratitude that I acknowledge the advice and technical expertise of Professor Anne French and her contribution to this study.

Acknowledgement and thanks goes to Dr Fleur James for her technical guidance particularly in the early stages of this study.

My thanks also to Drs Jenny Mills, Sue Beetson and Tibor Gaal, for their valued clinical pathology support and Renee Myles and Gavin D'Mello for their help processing the many laboratory samples generated as part of this project.

I acknowledge with gratitude the financial support of Boehringer Ingelheim.

Thanks are also due to all the breeders and veterinarians who assisted in the recruiting process of the dogs and the participating Cavaliers & their owners.

Finally, I wish to record the gratitude I feel towards my family and friends, for their unfailing support and encouragement over a prolonged period.

Dedication

For my family, Sally, Samantha and Judy,
always in my heart wherever we are

And in loving memory of my father, Garry David,
who knew wisdom, wonder and generosity of spirit,
you still inspire me each and every day

List of tables

List of figures

List of abbreviations

ACVIM	American College of Veterinary Internal Medicine
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APCC	Automated platelet clump count
ARJ/LAA	Area of the regurgitant jet relative to the left atrial area
BTG	β-thromboglobulin
CVHD	Chronic valvular heart disease
CKCS	Cavalier King Charles spaniel
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
GP	Glycoprotein
HCT	Haematocrit
HPF	High power field
IQR	Interquartile range
LA:Ao	Left atrial to aortic ratio
LVDDN	Left ventricular diameter during diastole, normalised to bodyweight
LVDSN	Left ventricular diameter during systole, normalised to bodyweight
MPC	Mean platelet component
MPM	Mean platelet mass
MPV	Mean platelet volume
MUVH	Murdoch University Veterinary Hospital
MVP	Mitral valve prolapse
MVR	Mitral valve regurgitation
NYHA	New York Heart Association
PCDW	Platelet component distribution width
PCT	Plateletcrit
PDW	Platelet volume distribution width
PFA	Platelet function analyser
PF4	Platelet factor 4

PISA	Proximal isovelocity surface area
PLT-I	Impedance platelet count
PLT-O	Optical platelet count
PMDW	Platelet mass distribution width
QBC	Quantitative buffy coat
SD	Standard deviation
TPO	Thrombopoietin
VWF	von Willebrand factor

1 Literature review

1.1 Background

Chronic valvular heart disease (CVHD), characterised by progressive myxomatous degeneration and thickening of the mitral valve leaflets, is the most common heart disease in dogs (Sisson et al. 1999). This disease is particularly common in CKCS, with echocardiographic evidence of mitral valve prolapse (MVP) reported in more than 80% of CKCS aged one to three years (Pedersen et al. 1999a).

Decreased platelet number and increased platelet size (thrombocytopenia and macrothrombocytosis, respectively) are also common in the CKCS (Eksell et al. 1994, Smedile et al. 1997, Pedersen et al. 2002, Singh and Lamb 2005). Previous studies report the prevalence of thrombocytopenia (defined as a platelet count less than 100 x 10^9/L) as 31-56% (Eksell et al. 1994, Olsen et al. 2001, Pedersen et al. 2002, Cowan et al. 2004, Olsen et al. 2004, Singh and Lamb 2005), with macrothrombocytosis (defined as greater than 30% of platelets with diameter ≥ 3μm or greater than 30% of platelets subjectively as large as or larger than a red blood cell) present in an overlapping 30-33% of dogs (Cowan et al. 2004, Singh and Lamb 2005).

In all species, valvular heart disease has the potential to affect platelet activation or function as a result of turbulent high-velocity blood flow and fluid shear stress (Brown et al. 1975). Increased platelet activation and reactivity would be expected initially; however, continuous stimulation and stress may lead to structural and biochemical changes associated with decreased platelet function (Tanaka and Yamane 2000). Alteration of platelet function in humans with heart disease contributes to the development of vascular remodelling, thromboembolic events, and fatalities (Michelson 2004).

There are conflicting reports regarding platelet activation and function in dogs with CVHD. A significant increase in closure time has been described in CKCS with moderate to severe mitral valve regurgitation (MVR) compared to those with minimal or mild regurgitation or healthy control dogs (Tarnow et al. 2003, 2004, 2005). A significant inverse relationship has also been identified between plasma von Willebrand factor (VWF) concentration and regurgitant jet size, as well as a relative absence of high-molecular-weight VWF multimers in samples obtained from dogs with moderate to severe MVR (Tarnow et al. 2004). Consequently, the prolonged closure time was thought to be a result of these quantitative and qualitative changes in VWF rather than an alteration of intrinsic platelet function. However, concurrent alteration of platelet function or platelet activation could not be excluded.

Enhanced platelet-leukocyte aggregation, which is suggestive of platelet activation, has been identified in dogs with congestive heart failure, compared with results for age-matched control dogs (Tarnow et al. 2010a). In addition, decreased platelet lifespan has been reported in dogs following experimental induction of MVR, which would support the presence of younger platelets with increased activity (Tanaka et al. 2002). However, plasma concentration of thromboxane B2, platelet surface P-selectin expression, and thromboelastographic variables are not significantly different between CKCS with CVHD and moderate to severe MVR, compared with results for affected dogs with absent or minimal MVR or healthy control dogs of other breeds (Tarnow et al. 2005, Tarnow et al. 2010b).

Aggregometric studies (Tanaka and Yamane 2000, Olsen et al. 2001, Tarnow et al. 2005) have also yielded conflicting results, with increased, decreased, and unchanged responses described in CKCS with advanced disease, compared with results for those with mild or no disease or healthy dogs of other breeds. Reasons for these discrepancies are unclear, but they may be explained by differences in laboratory methods, lack of standardisation of laboratory techniques, or differences in criteria for the classification of disease severity or control dogs. In addition, marked interbreed variability has been observed for the maximal aggregation

response (Cowan et al. 2004, Nielsen et al. 2007, Moesgaard et al. 2009) and breed composition of control groups has differed among studies. Therefore, in contrast to humans, the extent of platelet activation in dogs with CVHD remains uncertain.

Mean platelet component (MPC) concentration, which is measured by use of an optical-based haematology analyser, has emerged as a marker of platelet activation (Moritz et al. 2005). The MPC concentration is an estimate of platelet density, and decreased values are indicative of platelet activation (Ahnadi et al. 2004). In dogs, decreased MPC concentrations have been detected after exercise, in patients with inflammatory disease, and in association with immune-mediated thrombocytopenia (Moritz et al. 2003, Moritz et al. 2005, Bauer et al. 2012, Smith et al. 2014). It has been suggested that decreased MPC concentration is a more sensitive marker of platelet activation compared to P-selectin expression, in part because of the persistence of decreased MPC concentrations following activation despite the loss of cell-surface P-selectin (Macey et al. 1999, Moritz et al. 2005). The standard deviation (SD) of the platelet component concentration, known as the platelet component distribution width (PCDW), can also be calculated. This index is increased if both degranulated and non-degranulated circulating platelets are present, and it is considered another marker of platelet activation (Moritz et al. 2005). It is possible that MPC concentration and PCDW could provide additional information regarding the activation of platelets in dogs with heart disease.

This chapter will review CVHD in the CKCS and its similarities to MVP in people; clinical and echocardiographic indices of heart disease severity; methods of platelet number, activation and function assessment (with reference to previous findings in the CKCS); automated platelet indices (including MPC concentration and PCDW) and the reported associations between selected platelet indices and valvular heart disease.

1.2 Chronic valvular heart disease in the Cavalier King Charles spaniel and other dog breeds

1.2.1 Occurrence, pathology, inheritance and diagnosis

Chronic valvular heart disease, defined as progressive myxomatous degeneration and thickening of the mitral valve leaflets, is the most common heart disease in dogs (Sisson et al. 1999, Borgarelli et al. 2008) and is estimated to account for 75–80% of all heart disease cases in dogs (Häggström and Pedersen 2005).

Mitral valve prolapse is a fundamental and early feature of CVHD, and is defined as the protrusion of one or both valve leaflets to the atrial side of the plane of the mitral annulus during systole (Pedersen et al. 1999a). The degree of MVP correlates with the severity of MVR (Pedersen et al. 1996).

Chronic valvular heart disease has a strong small-breed predisposition. It is particularly common in the CKCS and many dogs are affected from a young age. In Denmark, ultrasonographic evidence of MVP was present in 82% (54/66) of CKCS between one and three years of age and 97% (84/87) of CKCS greater than three years of age, with a left apical systolic murmur identified clinically in 50% of dogs by 6.2 years (Pedersen et al. 1999a). In a French study, there was ultrasonographic evidence of CVHD in 91% (139/152) of CKCS of all ages, and a left apical systolic murmur in 50% of dogs by 6.6 years (Chetboul et al. 2004). Similarly, an American study reported the presence of a left apical systolic murmur in 56% (220/394) and 100% (10/10) of CKCS aged greater than four and 10 years, respectively (Beardow and Buchanan 1993). The latter study also reported that the age of referral for assessment of CVHD was lower in the CKCS with a mean age of 6.25 years compared to a mean age of 12 years in other breeds.

The pathological changes of CVHD are well described and are characterised by expansion of the extracellular matrix with glycosaminoglycans and

proteoglycans, disruption or loss of the collagen-rich fibrosa layer, and change in valvular interstitial cell phenotype (Kogure 1980, Pedersen and Häggström 2000). Morphologically, these changes result in enlargement and thickening of the mitral valve leaflets, inter-chordal hooding, elongation of the chordae tendineae and MVP (Pomerance and Whitney 1970, Pedersen et al. 1995). Collectively, these changes result in valve leakage during systole (MVR) (Whitney 1974). As CVHD progresses, an increase in left ventricular filling pressures associated with volume overload may result in left atrial and later ventricular dilation (i.e. eccentric hypertrophy) (Kvart et al. 2002). The progression of CVHD is inevitable but typically slow. In some cases, left-sided congestive heart failure will develop (Lord et al. 2010).

The cause of CVHD in dogs is not known; however, a genetic component is suspected based upon the higher prevalence, and results of genealogical and genetic studies, in specific breeds (Lewis et al. 2011, Madsen et al. 2011). Traditionally CVHD has been considered a complex polygenic threshold trait; a theory supported by more recent failure to identify association with any single major locus in genome-wide association studies (Madsen et al. 2011, French et al. 2012). Although traditionally described as a progressive, non-inflammatory disease, in recent years it has become apparent that canine CVHD is associated with immune system activation. A 2006 study evaluated genomic expression patterns from the anterior mitral valve leaflet of four dogs with severe CVHD using an oligonucleotide microarray, and identified uniform activation of several pathways including those involved in immunity and inflammation (Oyama and Chittur 2006). Based upon this finding, it is possible that the immune system is involved in the development and/or progression of CVHD.

A diagnosis of CVHD is commonly based on a combination of cardiac auscultation and two-dimensional, M-mode and colour-flow Doppler echocardiography (Muzzi et al. 2003). In brief, auscultation of a left-sided apical systolic murmur is consistent with MVR; two-dimensional and M-mode echocardiography permits identification of abnormal mitral valve thickening and prolapse and cardiac remodelling; and colour-flow Doppler

echocardiography enables estimation of the mitral regurgitant jet area, usually expressed as a percentage of the left atrial area (Pedersen et al. 1999a, Muzzi et al. 2003).

1.2.2 Disease-related variables of chronic valvular heart disease

1.2.2.1 Clinical and echocardiographic indices of mitral valve regurgitant jet severity

Murmur intensity/grade

Cardiac murmur intensity is most commonly graded using a six point grading system, with grades I-IV/VI varying in intensity, and grades V/VI and VI/VI characterised by the presence of a precordial thrill and a murmur that is audible with the stethoscope lifted off the chest wall, respectively. The severity of MVR is positively associated with heart murmur grade on auscultation (Ljungvall et al. 2009) and with severity of CVHD (heart failure class) (Häggström et al. 1995).

Colour-flow Doppler echocardiography

The assessment of MVR severity in dogs with CVHD is important because mild MVR is clinically insignificant whereas severe regurgitation is haemodynamically consequential and associated with increased morbidity and mortality (Bonow et al. 1998).

There is lack of a true gold standard in the assessment of MVR severity, with inherent advantages and disadvantages of each proposed method (Zoghbi et al. 2003). The Proximal Isovelocity Surface Area (PISA) method, a Doppler technique commonly used in people to quantify (as opposed to semi-quantify) MVR has been reported to be an accurate ultrasonographic method of estimating the severity of MVR (Kittleson and Brown 2003, Gouni et al. 2007, Chetboul and Tissier 2012). The PISA method permits assessment of the flow rate through the regurgitant orifice, measurement of the regurgitant

volume, and calculation of the regurgitant fraction (Chetboul and Tissier 2012). The PISA method is not routinely used in dogs because it is time-consuming and technically demanding (Gouni et al. 2007). Other limitations of the PISA method are that there is reduced accuracy in the absence of a circular orifice and that it cannot be used in the presence of multiple jets (which can occur with severe CVHD) (Chetboul and Tissier 2012). Furthermore, recently, the PISA method has been shown to be unreliable in the presence of eccentric jets, which are found in the majority of myxomatous degenerative valvular disease cases (Sargent et al. 2015).

A simpler technique to assess MVR severity is the estimation of the mitral regurgitant jet size using colour-flow Doppler mapping. Estimation of the mitral regurgitant jet size is typically made from a left parasternal 4-chamber view. The method involves frame-by-frame analysis to identify and estimate the percentage of the left atrial area, as assessed by eye, occupied by the largest mitral jet (Figure 1.1) (Pedersen et al. 1999a, Pedersen et al. 1999b). A related, semi-quantitative measurement of mitral regurgitant jet size is the area of the regurgitant jet relative to the left atrial area (ARJ/LAA), also obtained from a left parasternal 4-chamber view. The latter method involves the calculation of the maximum ratio of the regurgitant jet area signal to the left atrial area, using colour-flow Doppler echocardiography and computerised planimetry (Muzzi et al. 2003, Chetboul and Tissier 2012). A disadvantage of both the mitral regurgitant jet size estimation and ARJ/LAA methods is the ability of several factors such as arterial blood pressure, left atrial pressure, jet orientation, gain settings and pulse repetition frequency to influence results (Zoghbi et al. 2003, Gouni et al. 2007).

Despite its limitations, the ARJ/LAA method has been shown to correlate with quantitative Doppler techniques including mitral regurgitant volume ($r = 0.81$, $p < 0.001$) (Muzzi et al. 2003), the effective regurgitant orifice area ($r = 0.79$, $p < 0.001$) (Muzzi et al. 2003) and regurgitant fraction ($r = 0.58$, $p < 0.001$) (Gouni et al. 2007) and is therefore a valid option for assessment of MVR severity in a clinical setting.

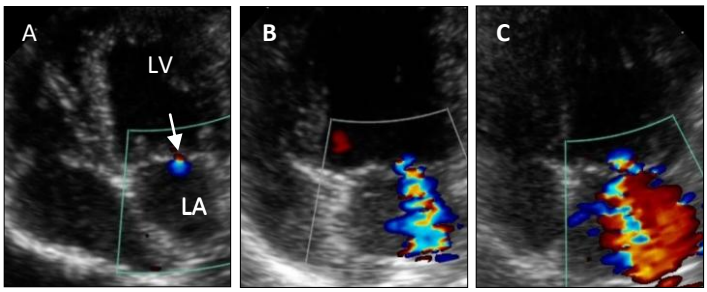

Figure 1.1: Mitral regurgitant jet size in 3 dogs with varying degrees of severity of CVHD.

Left parasternal 4-chamber view. In Image **A** the regurgitant jet (arrow) is estimated to occupy less than 15% of the left atrial chamber, consistent with minimal MVR; in Image **B** the regurgitant jet is estimated to occupy 15% to 50% of the left atrial chamber, consistent with mild MVR and in Image **C** the regurgitant jet is estimated to occupy greater than 50% of the left atrial chamber consistent with moderate to severe MVR, as previously described (Tarnow et al. 2003). LA: left atrium. LV: left ventricle.

1.2.2.2 Echocardiographic indices of left heart remodelling

Persistent and haemodynamically significant MVR results in volume overload (Chetboul and Tissier 2012).

Volume overload is first characterised by an increase in left atrial size, which is commonly assessed using the left atrial to aortic ratio (LA:Ao), obtained from the right parasternal short-axis approach at the level of the aortic valves (Thomas et al. 1993, Chetboul and Tissier 2012) (Figure 1.2).

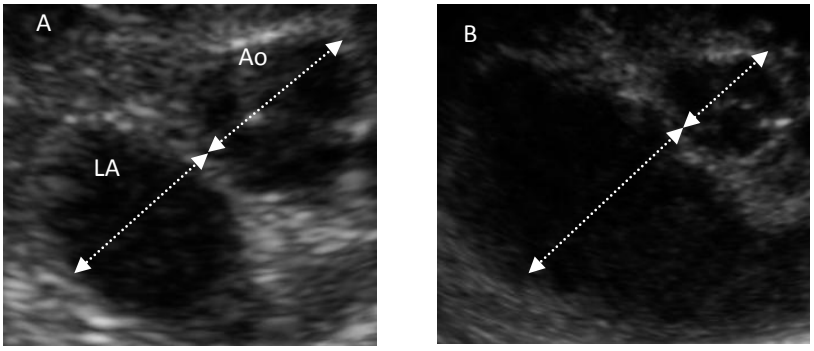

Figure 1.2: Left atrial to aortic ratios in 2 dogs with varying degrees of severity of CVHD.
Right parasternal short-axis view, at the level of the aortic valve as previously described (Thomas et al. 1993). Figure **A**: American College of Veterinary Internal Medicine (ACVIM) canine CVHD score A dog with a LA:Ao of 1; reference interval 1.03±0.09 (Hansson et al. 2002). Figure **B**: ACVIM canine CVHD score C dog with a moderately-markedly dilated left atrium (LA:Ao of 2).

With progression of MVR over time, there is a corresponding increase in volume overload that results in enlargement of the left ventricular chamber during end-diastole. This left ventricular eccentric hypertrophy, a marker of increased pre-load, may produce annular stretching and worsen the severity of MVR (O'Gara et al. 2008, Chetboul and Tissier 2012). The diameter of the left ventricle during diastole (referred to as the Left Ventricular Diameter during Diastole [LVDD]) is a commonly acquired index, obtained using M-mode echocardiography at the level of the chordae tendineae as guided by a 2-dimensional right parasternal short axis view (O'Gara et al. 2008, Chetboul and Tissier 2012).

The Left Ventricular Diameter during Systole (LVDS) is obtained using the same approach as LVDD, and can be used to assess for impaired systolic function in advanced CVHD cases (Chetboul and Tissier 2012). An increase in LVDS, in spite of enhanced left ventricular ejection into a low pressure left atrium during systole, is consistent with systolic myocardial dysfunction (Chetboul and Tissier 2012).

Left atrial size and left ventricular internal dimensions prior to, and at the onset of, congestive heart failure have been reported in 18 CKCS (Lord et al. 2010). Before the onset of congestive heart failure there was a slow increase in left atrial size, LVDD and LVDS until approximately 6-12 months prior to the onset of congestive heart failure, at which time there was a rapid rate of change suggesting that the rate of increase in left chamber size may be used to predict cardiac decompensation (Lord et al. 2010).

1.2.3 Comparative features of canine chronic valvular heart disease and mitral valve prolapse in people

It has been proposed that canine CVHD may be a suitable animal model for human MVP (Pedersen and Häggström 2000).

Mitral valve prolapse in people has similar macroscopic and microscopic pathological features to CVHD in dogs (Pomerance and Whitney 1970). Mitral valve prolapse is a fundamental feature of both diseases (Pedersen and Häggström 2000). Both CVHD and MVP have a suspected genetic component, which is considered to be autosomal or polygenic in people (Devereux et al. 1982, Wilcken 1992) and polygenic in the dog (Häggström et al. 1992). In both species, there is an increasing prevalence with age (Whitney 1974, Davies et al. 1978) and males are at higher risk than females of developing severe disease with increasing age (Davies et al. 1978, Swenson et al. 1996). There is a MVP disease association with a small stature and narrow chest in both dogs and people (Schutte et al. 1981, Olsen et al. 1999). Furthermore both MVP and CVHD have a slowly progressive disease course (Pedersen and Häggström 2000).

1.3 Platelets and platelet function

1.3.1 Platelet review

1.3.1.1 Platelet function in primary haemostasis

The control of haemorrhage (haemostasis) is essential for maintenance of life. Haemostasis is mediated through a complex series of pathways culminating in the development of a platelet plug (primary haemostasis) followed by the deposition of a stable fibrin clot (secondary haemostasis).

Platelets, or thrombocytes, are disc-shaped, anucleate cytoplasmic fragments that play an essential role in primary haemostasis and maintenance of vascular integrity (Barger 2003, Drachman 2004).

There are three main components to primary haemostasis that occur simultaneously: adhesion, activation and aggregation (Kelley 2013). Primary haemostasis is complex and a brief overview is provided below.

Adhesion

Following vessel damage, circulating platelets contact exposed subendothelial collagen and membrane-expressed tissue factor (Tvedten 2012). Next, the glycoprotein (GP), VWF, assists the adhesion of platelets to subendothelial collagen and to each other (Tvedten 2012). Additionally there is direct binding of platelets to subendothelial collagen via the integrin α2β1 and GP VI receptors (Brass et al. 2013, Kelley 2013).

Activation

Adhesion of platelets to subendothelial collagen initiates platelet activation (Kamath et al. 2001). There is activation of the platelet surface collagen receptor GP IIb-IIIa, platelet shape change from disc to sphere and

pseudopod formation, and release of stored contents from alpha and dense platelet granules (refer to Section 1.3.1.2). With activation, platelets synthesise and release thromboxane A2. Thromboxane A2 and alpha and dense granule contents attract more platelets to the growing platelet plug (Tvedten 2012, Brass et al. 2013). Platelet signalling mediates platelet shape change, promoting platelet spread over exposed subendothelial collagen (Kelley 2013).

Aggregation

Platelet aggregation is enhanced due to binding of plasma VWF, fibrinogen, and fibrin to activated GP IIb-IIIa (Brass et al. 2013). Aggregation of platelets leads to formation of the platelet plug and control of haemorrhage.

An abnormality at any stage in the fibrin clot formation process can result in failed haemostasis (Kelley 2013)*.*

1.3.1.2 Platelet structure

Platelet structure is complex and may can be divided into four sections: the peripheral zone, the structural zone, the organelle zone and the membranous system (Kelley 2013, White 2013).

The peripheral zone forms the most external platelet layer (Kelley 2013, White 2013). This section contains the outer platelet membrane, which is comprised of a phospholipid bilayer interspersed with specialised proteins and GP receptors integral to platelet function (Boudreaux 2008). The previously mentioned GP VI and GP IIb-IIIa are examples of GP located within this bilayer. Glycoprotein IIb-IIIa is the most common GP complex expressed on the surface of platelets (Boudreaux 2008).

The structural zone of the platelet is located beneath the peripheral zone and contains structures required to maintain the disc-shaped form of quiescent

platelets during circulation, and change platelet shape during platelet activation (Kelley 2013, White 2013). Broadly speaking, the structural zone comprises both microtubules and a cytoskeletal network. Microtubules are defined as hollow, cylindrical structures, comprising protofilaments with α-β tubulin dimer composition. Beta-1 tubulin is the most common tubulin isoform and is primarily expressed in megakaryocytes and platelets (Boudreaux 2008). A mis-sense mutation in the gene encoding β1-tubulin is responsible for macrothrombocytopenia in the CKCS (refer to Section 1.3.7.2.3). The cytoskeletal network, including actin and myosin, is responsible for microtubule contraction (and therefore assists with platelet shape change and proplatelet formation), granule movement and release, and thrombus retraction during platelet activation (Kelley 2013).

The organelle zone, located beneath the structural zone within the platelet cytoplasm, contains mitochondria, glycogen stores and storage granules (of which there are three main types: alpha, dense and lysosomal) (Boudreaux 2008, Kelley 2013, White 2013). Alpha granules are the most sizeable and numerous, and contain proteins including β-thromboglobulin (BTG), platelet factor 4 (PF4), fibrinogen and VWF (Boudreaux 2008). Additionally alpha granules also contain the trans-membrane proteins GP IIb-IIIa and P-selectin (Boudreaux 2010a). Dense granule contents include adenosine diphosphate (ADP), GP IIb-IIIa and P-selectin, high concentrations of Ca^{2+} and serotonin (Boudreaux 2010a).

The fourth platelet zone is the membranous system comprising the canalicular and dense tubular system (Kelley 2013, White 2013). The canalicular system comprises a series of interconnecting channels opening to the platelet surface and permitting external release of platelet granule contents during platelet activation (Boudreaux 2008). The dense tubular system, derived from smooth endoplasmic reticulum is responsible for platelet prostaglandin synthesis and is a site of calcium storage (Boudreaux 2008).

1.3.1.3 Platelet production

Mature megakaryocytes produce platelets via the process of proplatelet formation (Kelley 2013). Megakaryocyte maturation, including endomitosis, and formation of the demarcation system must occur before proplatelet formation can begin (Boudreaux 2010c). The latter involves in-folding of the megakaryocyte plasma membrane to create platelet cisternae (Boudreaux 2010c).

The first stage of proplatelet formation involves centrosome disassembly and movement of microtubules to the cell cortex (Italiano and Hartwig 2013). Secondly, thick pseudopodia arise from a single megakaryocyte pole (Italiano and Hartwig 2013). There is subsequent lengthening of the thick pseudopodia to form thin proplatelet processes (Italiano and Hartwig 2013). Thirdly the sliding movement of microtubules within thick pseudopodia and thin proplatelet processes together with actin-myosin complexes forces mediate elongation of these proplatelet processes (Italiano and Hartwig 2013). Fourthly, within the tips of the proplatelet processes, microtubules loop upon themselves and re-enter the shaft to assemble at the proplatelet tip (Italiano and Hartwig 2013, Kelley 2013). The tips of the proplatelet processes, the main site of platelet assembly, is where microtubule coiling, a characteristic of circulating platelets, occurs (Kelley 2013).

Eventually the megakaryocyte cytoplasm resembles a mass of proplatelet processes (Italiano and Hartwig 2013). Mature megakaryocytes are located close to endothelial cells permitting proplatelet processes to reach marrow sinusoids (Boudreaux 2010b). Proplatelet processes are released into the blood stream, followed by release of individual platelets from the proplatelet ends (Italiano and Hartwig 2013).

1.3.1.4 Regulation of platelet production

Primary haemostasis requires an adequate circulating platelet mass (Kelley 2013). Platelet mass is defined as the volume of blood that is comprised

solely of platelets (refer to Section 1.3.3.3). Platelet mass is an indirect measure of the number of available thrombopoietin (TPO) receptors.

Platelet mass is maintained primarily by the TPO/TPO-receptor system (Kuter 1996, Kelley 2013). Thrombopoietin is a cytokine that is produced primarily by the liver and to a lesser degree the kidney (Boudreaux 2010b). Receptors are present on both megakaryocytes and platelets. When TPO binds to megakaryocytes thrombopoiesis occurs; however when TPO binds to platelets, there is internal degradation of TPO which renders it unavailable for binding to megakaryocytes (Kuter 1996, Kelley 2013). In health, platelet numbers are maintained within a narrow range, with the majority of TPO degraded by platelets and low basal concentrations maintaining platelet production (Kuter 1996, Kelley 2013).

With a decrease in platelet mass (which occurs with a decrease in the number of platelets or a decrease in the number/function of platelet TPO receptors), there is an increase in TPO concentration, with consequent megakaryocyte stimulation and enhanced thrombopoiesis (Kuter 1996, Kelley 2013).

Assuming there is consistent TPO receptor density, the number of receptors on numerous small platelets is equivalent to the number of platelet receptors on lesser numbers of large platelets. Thus platelet mass (and the number of TPO receptors), and not platelet number determines platelet production (Kuter 1996, Kelley 2013).

1.3.2 Thrombocytopenia

1.3.2.1 Causes of true thrombocytopenia

Thrombocytopenia, defined as a low number of platelets in the peripheral circulation, occurs with decreased or defective platelet production, increased

platelet consumption or loss, increased platelet destruction or abnormal distribution (sequestration) (as reviewed by Feldman et al., 2000).

Decreased or defective platelet production may result from drug, chemical or toxin exposure causing cytotoxicity or an idiosyncratic reaction; irradiation causing cell death and bone marrow suppression; viral or rickettsial infections; myelophthisis due to neoplasia or fibrosis; myelonecrosis; or genetic β-tubulin defects (Boudreaux 2010b). Increased platelet consumption relates to activation of the coagulation system and may occur for example in disseminated intravascular coagulation. Increased platelet loss may result from extensive trauma or external haemorrhage (Boudreaux 2010b). Increased platelet destruction most commonly occurs due to immune-mediated mechanisms. Immune-mediated destruction of platelets is primary (idiopathic) or secondary to infectious agents, neoplasia or drug administration (Boudreaux 2010b). Platelet sequestration occurs with diseases associated with splenomegaly or less commonly liver or bone marrow disease (Boudreaux 2010b).

1.3.2.2 Causes of artefactual thrombocytopenia

Inappropriate blood sample collection and handling techniques may induce *in vitro* platelet activation and platelet aggregation (Tvedten 2012).

Artefactual platelet aggregation may result in uneven distribution of platelets within a blood sample, and pre-analytical error, with all platelet counting methodologies (manual counting, blood smear estimate and automated counting) (Tvedten 2012). An artefactual decrease in platelet count i.e. pseudothrombocytopenia may result (Zelmanovic and Hetherington 1998).

Platelet activation may also occur during sample collection due to:

- blood sampling from small peripheral veins or veins with decreased blood flow
- blood sampling using small-bore needles or excessive syringe pressure
- excessive agitation of samples during mixing as opposed to gentle inversion
- overfilling of blood collection tubes or inadequate sample mixing with anticoagulant
- prolonged blood storage time (Pewarchuk et al. 1992).

In addition, *in vitro* platelet aggregation occurs in certain individuals when blood contacts particular anticoagulants (Boudreaux 2010b). Ethylenediamine tetra-acetic acid (EDTA)-dependent pseudothrombocytopenia is well described in humans (Bizzaro 1995), with only rare (single case reports) in the dog (Wills and Wardrop 2008) , horse (Hinchcliff et al. 1993) and miniature pig (Ragan 1972). In people, EDTA-induced pseudothrombocytopenia involves autoantibody-mediated agglutination of platelets (Casonato et al. 1994). EDTA induces calcium chelation and promotes a conformational change in GP IIb/IIIa on platelets, leading to exposure of a cryptic antigen with subsequent anti-platelet antibody binding and platelet aggregation (Casonato et al. 1994, Fiorin et al. 1998).

Traditionally, it was believed that platelet clumping rarely occurred with other anticoagulants, including citrate (Shreiner and Bell 1973, Onder et al. 1980, Payne 1985), however, results of more recent studies appear to contradict this assumption. Lower platelet counts (due to increased platelet aggregation) have been reported in citrated human blood samples compared to EDTA-anticoagulated samples (Macey et al. 1999, Ahnadi et al. 2003). Likewise, Stokol and Erb (2007) reported that the median platelet count in 50 dogs with neoplasia was significantly lower in citrate- compared to EDTA-

anticoagulated blood, due to platelet aggregation occurring more frequently in the samples containing citrate.

1.3.2.3 Breed variation in platelet characteristics

Certain dog breeds have platelet indices that commonly lie outside non-breed specific reference intervals. In the CKCS, thrombocytopenia is common and is usually accompanied by macrothrombocytosis (Brown et al. 1994). A review of CKCS platelet characteristics is presented (refer to Section 1.3.7).

Other breed specific platelet variations include mild thrombocytopenia in greyhounds (Santoro et al. 2007) and thrombocytopenia and macrothrombocytosis in the Norfolk terrier (Gelain et al. 2010). The cause of the greyhound and Norfolk terrier breed specific platelet variations is currently unknown.

1.3.3 Methods of platelet enumeration

There are four major laboratory methods of counting platelets: 1) manual methods using microscopy, 2) automated methods using commercial analysers including a) impedance analysis and b) optical light scatter/fluorescence analysis, 3) directly measured plateletcrit (PCT) (from which a platelet count is derived) (Tvedten et al. 2008, Tvedten et al. 2012) and 4) immunoplatelet counting by flow cytometry (Harrison and Briggs 2013).

1.3.3.1 Manual methods

Manual methods of assessing platelet number are manual counting and blood smear estimation by microscopy (Briggs et al. 2007).

Until recently, manual counting was considered the gold standard in people (Lancé et al. 2012). With this method, a glass counting chamber (haemocytometer) set on top of a microscope stage is used to count the number of platelets in a specific fluid volume (after the addition of erythrocyte-lysing agents), permitting calculation of the concentration of platelets in the fluid (Tvedten 2012). Manual counting however is laborious and subjective with a high degree of imprecision. Inter-observer coefficient of variation is typically reported to be approximately 10-25% (Harrison et al. 2000, Briggs et al. 2007).

The second manual method, blood smear estimation, involves estimation of platelet numbers during examination of a stained blood smear (Tvedten 2012). Blood smear estimates, compared to manual counts, are obtained comparatively simply and quickly and provide a reasonable estimate of platelet numbers (Tvedten 2012).

1.3.3.2 Automated methods

Automated cell counting is based on either impedance or optical methodology (Harrison and Briggs 2013).

Impedance

The impedance principle (or Coulter principle) transformed blood counting in the 1950s, and in the late 1970s impedance-based automated platelet counts became commercially available (Harrison and Briggs 2013). Today it remains a common method in many modern instruments but has largely been replaced by optical counters.

According to the impedance principle, cells are passed through an aperture in an electrode in an electrically conducting fluid. The passage of a cell produces a change in electrical resistance, proportional to the size of the cell (Tvedten 2012). Cell number and size are determined by the frequency and

magnitude of change in resistance, respectively. Impedance counters are calibrated to count cells within an appropriate species specific size interval via adjustment of electronic thresholds (Tvedten 2012). The presence of larger platelets may lead to analytical inaccuracy because macrothrombocytes can be misclassified as erythrocytes (Moritz and Becker 2010, Tvedten 2012). This is a recognised problem in the CKCS breed (refer to Section 1.3.7.1.2).

Optical

Optical based cell counting methods were introduced in the 1970s (Lancé et al. 2012). Optical counters measure not only the size (volume) but also internal complexity of cells, based on assessment of light scatter at different angles (refractive index) or cell fluorescent intensity. This has reduced the analytical error associated with discrimination of cells based upon volume alone (Moritz and Becker 2010).

Optical based haematology instruments used in referral veterinary laboratories include the Advia 120/2120 (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) and Sysmex XT-2000iV (Sysmex Corporation, Kobe, Japan) (Tvedten 2012).

Advia 120/2120

The Advia 120/2120 uses the flow cytometer principle and contains a laser optical bench that consists of a flow cell, laser diode, and detector (Macey et al. 1999). The Advia 120/2120 differentiates platelets from other cells based on cell size (volume) and refractive index (density) (Macey et al. 1999).

After dilution, blood cells are passed through a flow cell that is illuminated by a laser diode. The resultant light scatter signals are acquired at 2 different angles: 2-3° (low angle scatter) and 5-15° (high angle scatter) (Briggs et al. 2007). These values are translated into volume and refractive indices by calculation, using the Mie theory of scattering of light (Brummitt and Barker

2000). Two light-scatter measurements are produced, and are displayed on a cytogram with platelets identified in the region corresponding to a volume of 1 to 60 fL and refractive index of 1.35 to 1.40. The platelet scatter cytogram (Figure 1.3) displays platelets with volumes of 0 fL to 20 fL and large platelets 21 to 30 fL, whereas the large platelet area of the red blood cell scatter cytogram (Figure 1.4) displays large platelets with volumes between 31 fL and 60 fL (Siemens 2002). The final platelet count is the sum of all platelets and large platelets, detected in the platelet and red blood cell scatter cytograms respectively (Briggs et al. 2007).

Integrated analysis is used to assist discrimination of standard-sized platelets, large platelets, erythrocytes, erythrocyte fragments and erythrocyte ghosts (Brummitt and Barker 2000, Briggs et al. 2007). Erythrocyte fragments have a higher refractive index than platelets and as a result are located low and to the right of the graph, whereas red cell ghosts have a refractive index less than platelets and as a result are located above and to the left (Briggs et al. 2007).

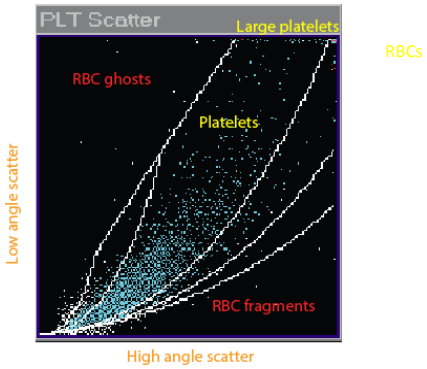

Figure 1.3: Platelet scatter cytogram.
A graphical display of different light scatter signals with high-angle on the x axis, and the low-angle on the y axis. The platelet scatter cytogram depicts platelets (volumes of 0 fL to 20 fL), large platelets (21 to 30 fL), erythrocytes and erythrocyte ghosts and fragments. Graph reproduced with permission (copyright of Siemens).

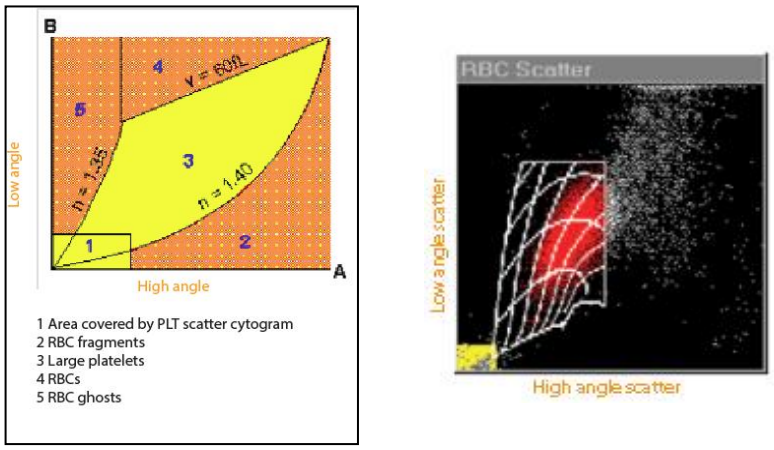

Figure 1.4: Red blood cell scatter cytogram.
Area labelled area 3 depicts large platelets with volume 31 to 60 fL. Area labelled 1 is the platelet scatter cytogram (refer to figure 1.3). Graph reproduced with permission (copyright of Siemens).

Sysmex XT-2000iV

By contrast to the Advia 120/2120 which uses size (volume) and refractive index (density) to identify platelets, the Sysmex XT-2000iV uses size and nucleic acid staining (Tvedten 2012).

The Sysmex XT-2000iV provides both an optical and an impedance platelet count (PLT-O and PLT-I respectively). The optical platelet count is obtained using the reticulocyte channel. Firstly, the RNA/DNA of platelet membranes and granules and reticulated cells are stained with a fluorescent dye (polymethine). Secondly, each cell is passed through the flow cell illuminated by a laser diode. The fluorescent intensity of each cell is determined, and permits the differentiation of platelets and large platelets from erythrocytes and reticulocytes (Tvedten 2012).

The majority of large automated haematology analysers are developed for use in people, with species adaptation necessary for animal use (Moritz et al. 2004). Species adaptation is achieved with software packages used to modify analyser settings (Moritz et al. 2004). Table 1.1 lists the platelet counting principle of some automated haematology instruments that have been assessed in the veterinary literature.

Manufacturer	Instrument	Platelet counting principle
Siemens	Technicon H1	Optical (single scatter channel) (Bertazzolo et al., 2007)
	Advia 120	Optical
	Advia 2120	Optical (Tvedten et al., 2012; Kelley et al., 2014; Davies et al., 2008)
Sysmex corporation	Sysmex XT-2000iV	Impedance and optical (Willard and Tvedten 2011)
Abbott diagnostics	Cell-Dyn 3500* *Later Cell-Dyn models including the Cell-Dyn 4000 and Cell-Dyn Sapphire provide impedance, optical and immunological counts	Impedance (Tvedten et al., 2008)

Table 1.1: Summary of some of the automated haematology instruments assessed in the veterinary literature for their ability to discriminate platelets from other cells.

1.3.3.3 Plateletcrit

Platelet count can also be derived from PCT. Plateletcrit refers to the volume of blood that is comprised solely of platelets, and is reported as a percentage (Tvedten 2012).

Importantly, PCT is the most physiologically relevant platelet parameter (Northern and Tvedten 1992, Butkiewicz et al. 2006) because platelet function depends on total platelet mass rather than platelet number (Thompson and Jakubowski 1988, Smedile et al. 1997). Plateletcrit is measured by two different methods, the direct method (from which a platelet count is derived) or the calculated method.

Direct method

With the direct method, the IDEXX VetAutoread Hematology analyser (IDEXX Laboratories, Westbrook, ME, USA) is used to perform a Quantitative Buffy Coat (QBC) analysis. Plateletcrit is measured directly from the width of the platelet layer in QBC tubes filled with blood and centrifuged (similar to the way in which haematocrit [HCT] is assessed). The VetAutoread converts the PCT to a platelet count (platelets/L), an index that is more familiar to clinicians. The actual method by which the VetAutoread converts PCT to platelet count is not publicly available (Tvedten et al. 2012).

Calculated method

The Advia 120/2120 reports a calculated PCT which is derived from the product of the platelet count and mean platelet volume [MPV] (Tvedten et al. 2012). The PCT reported by the Advia 120/2120 is expressed as a percentage.

The Sysmex XT-2000iV on the other hand, uses the impedance channel to determine MPV (and thus PCT), and is therefore unable to provide an accurate PCT in cases with macrothrombocytopenia (Lilliehöök and Tvedten 2009).

1.3.3.4 Immunoplatelet counting by flow cytometry

Immunoplatelet counting by flow cytometry is the current platelet counting reference method for people (Harrison et al. 2000). With this method, monoclonal antibodies against specific platelet cell surface antigens are conjugated to a fluorophore and antigens are detected by their fluorescence as they pass through a flow cytometer (Harrison et al. 2000). This technique is currently not used in veterinary practice, and will not be discussed further.

1.3.4 Additional platelet indices provided by the Advia 120/2120

In addition to the commonly reported platelet count, the Advia 120/2120 provides additional platelet indices including MPV, platelet volume distribution width (PDW), MPC concentration, PCDW, mean platelet mass (MPM), platelet mass distribution width (PMDW) and platelet clump and large platelet indices (Tvedten 2012). The meaning of these platelet indices are briefly described below and summarised in Table 1.2.

Several of these indices have been proposed to be markers of platelet activation. During activation, platelets swell due to fluid uptake and degranulate (Macey et al. 1999) resulting in an increase in MPV and a decrease in MPC concentration and MPM (Park et al., 2002). This is accompanied by an increase in the distribution width of each index (PDW, PCDW and PMDW) (Chapman et al. 2003, Stokol and Erb 2007).

Platelet parameter	**Abbreviation**	**Unit**	**Description**
Platelet count	PLT	10^9/L	Platelet count
Plateletcrit	PCT	%	Volume of blood comprised of platelets
Mean Platelet Volume	MPV	fL	Average platelet size
Platelet Volume Distribution Width	PDW	%	Indicator of the variation in platelet size
Mean Platelet Component Concentration	MPC concentration	g/L	Measure for platelet refractive index, linearly related to platelet density
Platelet Component Distribution Width	PCDW	g/L	Indicator of the variation in platelet density
Mean Platelet Mass	MPM	pg	Measure for mass of platelet component
Platelet Mass Distribution Width	PMDW	pg	Indicator of the variation in platelet component mass
Automated Platelet Clump Count	APCC	+ (if APCC is > 300)	Automated flag of platelet aggregates
Large platelet	LPLT	+ (%LPLT 10 to 11.9%) ++ (%LPLT 12 to 14%) +++ (%LPLT > 14%)	Platelets with a volume 21 to 60 fL

Table 1.2: Major Advia 120/2120 platelet indices

1.3.4.1 Mean platelet volume

Mean platelet volume refers to the average volume of platelets (Bommer et al. 2008).

An increase in platelet size may be real or artefactual:

As summarised previously, an increase in MPV occurs with platelet activation (Park et al. 2002). However, MPV may increase for a variety of other reasons. Unsurprisingly, breed-associated macrothombocytosisis is associated with an increased MPV (Tvedten et al. 2012). Additionally, an increased MPV occurs with accelerated thrombopoiesis (for example in cases with increased platelet consumption/loss or increased platelet destruction) (Brown et al. 1994, Wiwanitkit 2004, Boudreaux 2010b).

Anticoagulant type, storage time, temperature and osmotic environment are also reported to affect platelet size and shape (Boudreaux 2010b). It has been suggested that only citrated canine blood samples are suitable for assessment of platelet size because platelets exposed to EDTA undergo an artefactual increase in size as assessed by light microscopy (Handagama et al. 1986). Although a later study in 69 healthy CKCS showed that macrothrombocytosis (defined as greater than 30% of platelets having a diameter of at least 3 μm on microscopic examination) was consistently identified in both EDTA and citrated samples of 22 dogs, agreement between MPV in both samples was not assessed (Cowan et al., 2004).

1.3.4.2 Platelet volume distribution width

Platelet volume distribution width provides an indicator of the variation in platelet size (Russell 2010). In order to calculate the PDW, the SD of platelet volume is divided by the MPV and multiplied by 100 (Brummitt and Barker 2000). In humans, PDW has been used to differentiate between destructive thrombocytopenia (immune-mediated thrombocytopenia) and hypoproductive thrombocytopenia (aplastic anaemia), for which there is an increase and

decrease in PDW respectively (Kaito et al. 2005). Despite the potential use of PDW and MPV in dogs, they are currently not routinely used in veterinary practice. Published reference intervals for PDW and MPV are available for dogs (Moritz et al. 2004), however due to the non-linear inverse relationship of these volume indices with platelet count, interpretation of changes in PDW and MPV requires consideration of an individual's platelet count (Bommer et al. 2008).

1.3.4.3 Mean platelet component concentration and platelet component distribution width

Mean platelet component concentration is an automated platelet index, provided by the Advia 120/2120 (Macey et al. 1999). It is a measure of platelet refractive index, which is linearly related to platelet density, and inversely related to platelet activation (Ahnadi et al. 2004).

Platelet component distribution width is an indicator of the variation in MPC concentration and PCDW is increased if both non-degranulated and degranulated circulating platelets are present. An increased PCDW is therefore an indirect marker of platelet activation (Moritz et al. 2005, Boudreaux 2010b).

The calculation of MPC concentration is based upon the following formula: MPC concentration (g/dL) = (mean refractive index -1.333)/(0.0018/(g/dL)), where 1.333 = refractive index of water and 0.0018 (g/dL) = average refractive index increment (Chapman et al. 1998). The formula for PCDW is calculated as the SD of the platelet refractive index histogram (Siemens 2002).

Effects of factors other than platelet activation on MPC concentration have not been assessed in dogs. However, in one study involving 500 healthy people, MPC concentration increased from childhood (10-18 years) to adult age (18-45 years) and decreased thereafter (Giacomini et al. 2001). Macey et al. (1999) assessed changes in MPC concentration and P-selectin

expression in 20 human blood samples anticoagulated with EDTA, stored at room temperature, and assessed at timed intervals over a 3-hour period. The MPC concentration decreased and P-selectin expression increased over time. The relationship between age and MPC concentration, and the effect of storage time, in dogs is unknown.

Mean platelet component concentration studies in people

The MPC concentration has been used as an indicator of platelet activation in multiple human studies (Table 1.3).

Disease/physiologic state	**Result**	**Anticoagulant; time to analysis following venipuncture**	**Reference**
Congestive heart failure (due to left ventricular systolic dysfunction)	MPC concentration is lower in patients with congestive heart failure compared to healthy controls	Sodium citrate; not recorded	(Chung et al. 2007)
Coronary disease	MPC concentration is lower in patients with acute myocardial infarction compared to patients with unstable angina	EDTA; between 30-240 minutes	(Kennon et al. 2003)
Coronary disease	MPC concentration is lower in patients with stable angina, unstable angina and acute myocardial infarction compared to healthy controls	EDTA; immediately after collection	(Pawlus et al. 2010)
Diabetes mellitus	MPC concentration is lower in diabetes mellitus compared to healthy controls	EDTA; within 60 minutes	(Bae et al. 2003)
Strenuous exercise (pre- and post-race sampling)	Racing is associated with a decrease in MPC concentration	EDTA; within 90 minutes	(Kratz et al. 2006)
Deep vein thrombosis	MPC concentration is lower in patients with deep vein thrombosis compared to healthy controls	EDTA; within 60 minutes	(Cay et al. 2012)
Anti-platelet therapy (pre- and post-clopidogrel sampling)	Clopidogrel therapy is associated with an increase in MPC concentration	EDTA; within 60 minutes	(Ahnadi et al. 2004)

Table 1.3: Studies that have used MPC concentration as an indicator of platelet activation in people.
All reported results were statistically significant ($p < 0.05$).

Mean platelet component concentration studies in dogs

The use of MPC concentration as an indicator of platelet activation in dogs has been reported in three studies to date:

Two studies used MPC concentration to assess platelet activation after exercise. Moritz et al. (2003) reported a significant decrease in MPC concentration and increase in PCDW following short duration, strenuous, sled-pulling activity in 18 Siberian Huskies. By contrast, Bauer et al. (2012) reported no significant change in MPC concentration or PCDW following submaximal aerobic exercise in nine healthy beagles. These conflicting results may reflect differences in the type or duration of exercise, or be related to the smaller number of dogs (and lower power) in the latter study.

Moritz et al. (2005) compared MPC concentration and P-selectin expression for the identification of platelet activation in 20 dogs with septic or non-septic inflammatory diseases. The proportion of dogs with decreased MPC concentration (16/20) was higher than the number of dogs with increased P-selectin expression (13/20). Due to the lack of a gold standard, it was not known if MPC concentration was more sensitive or less specific compared to P-selectin expression as a marker of platelet activation. However, such conclusions must be interpreted with caution due to the low number of dogs in the latter study. The PCDW was greater than the upper limit of the reference interval in 14 dogs.

1.3.4.4 Mean platelet mass

Mean platelet mass is a measure of the mass of platelet component excluding water (Zelmanovic 2006). A decrease in MPM would be expected following platelet activation due to degranulation.

Mean platelet mass is obtained using conversion of volume and refractive index by mathematical algorithm:
MPM (in pg) = MPC concentration (in g/dL) x MPV (in fL) x 100 (Chapman et al. 1998).

1.3.4.5 Platelet mass distribution width

Platelet mass distribution width is an indicator of the variation in mass of platelet component excluding water.
The formula for PMDW is calculated as the SD of the platelet mass histogram (Siemens 2002).

1.3.4.6 Automated platelet clump count

Assessment of platelet clumping may be subjective (examination of the frequency and degree of platelet aggregation at the blood smear feathered edge) or quantitative (using the Advia 120/2120 APCC index).

The number of platelet clumps detected by the Advia 120/2120 corresponds to the number of events that fall within the platelet clump area, which originates from the noise cluster area in the peroxidase channel and extends to the right of the lymphocyte area (Siemens 2002, Stokol and Erb 2007). When the APCC is greater than 300, the Advia 120/2120 flags the sample by marking the platelet clumps index as '+' (Siemens 2002).

In a 2007 study that assessed APCC in dogs, the Advia 120/2120 flagged platelet clumps in 3 of 51 samples without visually detected clumps in smears (to provide a specificity of 94%) and failed to flag platelet clumps in 44 of 49 samples in which large or frequent aggregates were detected visually (to provide a sensitivity of 10%) (Stokol and Erb 2007). The results of this latter study therefore suggest that APCC is poorly sensitive and highly specific at detecting platelet clumping and that APCC should not be used

independently to verify the presence or absence of platelet clumps (Stokol and Erb 2007).

1.3.5 Assessing macrothrombocytosis

In the majority of dog breeds, platelets are typically one quarter to half the size of an erythrocyte, and only occasionally larger than a red blood cell (Boudreaux 2010b). The term macrothrombocyte refers to a platelet of increased size; and macrothrombocytosis refers to the presence of increased numbers of large platelets in circulation (Bertazzolo et al. 2007). Assessment of macrothrombocytosis may be subjective or quantitative:

- A platelet is subjectively defined as a macrothrombocyte if it is as large as or larger than a red blood cell (as assessed by light microscopy), and a diagnosis of macrothrombocytosis is made if more than 30% of platelets fulfil this criterion (Singh and Lamb 2005, Gelain et al. 2010).

- Macrothrombocytosis can be quantitatively assessed using electron microscopy (Cowan et al. 2004). By this method, a platelet is defined as a macrothrombocyte if it has a diameter greater or equal to 3µm, and a diagnosis of macrothrombocytosis is made if more than 30% of platelets fulfil this criterion (Cowan et al. 2004).

The number of macrothrombocytes may also be quantitatively assessed using an automated haematology instrument such as the Advia 120/2120 or Sysmex XT-2000iV. The Advia 120/2120 classifies platelets with a volume 21 to 60 fL as large, and flags the presence of large platelets in the sample using the large platelet index when greater than or equal to 10% of the platelets fall within this range (Table 1.4) (Siemens 2002). The Advia 120/2120 also reports the actual number of large platelets.

Advia 120/2120 large platelet index severity level	% Large platelets
+	≥ 10 to 11.9
++	12 to 14
+++	> 14

Table 1.4: Advia 120/2120 large platelet index severity level - percentage of large platelets in each category

The Sysmex XT-2000iV has also been used to determine macrothrombocyte count. In one study utilising the Sysmex XT-2000iV, a PLT-O cytogram was regated with a subjective algorithm based on the appearance of the PLT-O cytograms from dogs without large platelets, and this algorithm was then applied to all original PLT-O counts. The reported number of large platelets per sample was the difference between the original PLT-O count and the regated optical count (with the latter representing the number of normal sized platelets) (Tvedten et al. 2008).

1.3.6 Analysis of platelet function

The following tests of platelet function are based on different biologic principles, 1) platelet aggregometry, 2) CT, 3) thromboelastography and 4) platelet activation markers:

1.3.6.1 Platelet aggregometry; a low shear assay

Platelet aggregometry involves the assessment of platelet aggregation after exposure to certain agonists and provides an assessment of platelet function (Tvedten 2012). There is no standardised method for assessing platelet aggregation in dogs, with test procedures and aggregating substances varying between institutions and studies (Tanaka and Yamane 2000).

Optical (turbidometric) aggregometry

Optical aggregometry was introduced in the 1960s (Carr Jr 1997). Using this method, agonists are added to platelet-rich plasma to cause platelet aggregation (Jandrey 2012). As aggregation progresses the platelet-rich suspension clears, resulting in increased light transmission (Jandrey 2012). This alteration in light intensity is detected by a spectrophotometer and recorded digitally or by chart recorder. Results are reported as the percentage increase in light transmission relative to the pre-agonist baseline aggregation (Dosh and Steinhubl 2010). Disadvantages of this method are that platelets aggregate under low shear stress conditions and that the absence of other cellular elements (such as red and white blood cells) may result in suboptimal mimicry of *in vivo* platelet aggregation (Dosh and Steinhubl 2010). In addition, the need for centrifugation to create the platelet-rich plasma may predispose to platelet aggregation prior to analysis (Jandrey 2012). Despite these problems, optical aggregometry has been widely used in veterinary science (including CKCS platelet aggregation studies, refer to Section 1.4.1.1).

Whole blood impedance aggregometry

The technique of whole blood impedance aggregometry, introduced in the 1980s, may avoid some sample preparation issues associated with optical aggregometry (Jandrey 2012). Additionally, due to the use of whole blood, impedance aggregometry may be more physiologically appropriate than optical aggregometry (Jandrey 2012). With this technique two electrodes are placed in a sample containing anticoagulated whole blood and electrical impedance is assessed pre- and post- addition of a platelet agonist. During aggregation, platelets form a monolayer on electrodes and change (increase) the impedance of an electrical current (Jandrey 2012). The degree of aggregation is thus assessed as the increase in electrical impedance following addition of an agonist.

1.3.6.2 Platelet function analyser; a high shear assay

The platelet function analyser (PFA-100, Siemens Healthcare Diagnostics Product GmbH, Marburg, Germany), is a point of care, bench-top platelet function analyser developed for use in people and validated for use in dogs (Mischke and Keidel 2002). The PFA-100 is a sensitive screening tool for general defects in primary haemostasis (Clancey et al. 2009a).

The PFA-100 evaluates both platelet adhesion and aggregation by simulating high-shear blood flow at a site of endothelial damage (Callan and Giger 2001). This is achieved by rapid *in vitro* flow of a citrated whole blood sample through an aperture in a disposable collagen-coated membrane containing ADP or epinephrine (Tvedten 2012). The high shear blood flow causes platelet activation and adhesion to the membrane, mediated primarily by VWF (Tarnow and Kristensen 2010). The subsequent release of platelet granules and presence of agonist causes platelet aggregation via GP IIb-IIIa (Tarnow and Kristensen 2010). The time it takes for a platelet plug to form and occlude blood flow through the membrane aperture is measured in seconds and is called the CT (Tarnow and Kristensen 2010) (Figure 1.5). Epinephrine does not consistently cause platelet aggregation in dogs and collagen-epinephrine cartridges are therefore considered to be less accurate in the dog, and the use of collagen-ADP cartridges are more commonly recommended (Clemmons and Meyers 1984, Tvedten 2012).

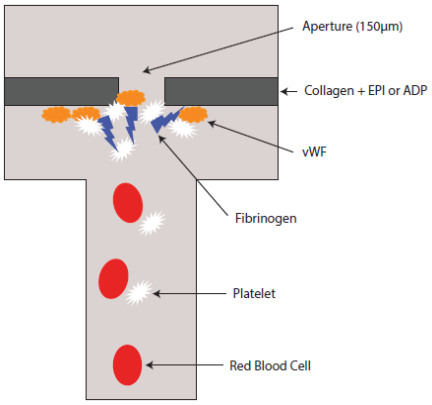

Figure 1.5: Cross-sectional diagram of the Platelet Function Analyser
(PFA-100) instrument (Siemens Healthcare Diagnostics Product GmbH, Marburg, Germany).
Blood is passed rapidly through an aperture in an agonist containing collagen-coated membrane to mimic *in vivo* platelet adhesion and activation. The time is takes for a platelet plug to form and occlude blood flow is the closure time (adapted from Jandrey 2012).

The PFA-100 CT is sensitive to platelet function, platelet number, HCT and drug therapy:

In the absence of macrothrombocytosis, there is an inverse relationship between CT and platelet count less than 150 x 10^9/L (Mischke and Keidel 2003).

Additionally in dogs there is an inverse relationship between CT and HCT (Mischke and Keidel 2003). Reasons for the latter include increased blood flow (associated with decreased blood viscosity) and altered blood cell distribution (more central flow of platelets within *in vivo* and *in vitro* capillaries) with lower HCT values (Mischke and Keidel 2003). One study investigated the change in CT (using collagen-ADP cartridges) following haemodilution of whole blood samples using fresh autologous platelet-rich plasma. The initial samples with HCT 0.39-0.54 L/L produced a mean CT +/- SD of 57.8 +/- 5.75 seconds, whereas the haemodiluted samples, with HCT

of 0.33 and 0.27 L/L, produced significantly prolonged CT of 61.1 +/- 4.64 seconds and 64.3 +/- 6.79 seconds respectively. Overall, an increase in CT has been demonstrated in dogs with a HCT less than 30-40% (Callan and Giger 2001, Mischke and Keidel 2003, Clancey et al. 2009b).

Furthermore, non steroidal anti-inflammatory drug administration (specifically aspirin and ketoprofen therapy) has been shown to increase CT in dogs when collagen-ADP and/or collagen-epinephrine cartridges are used (Keidel and Mischke 1998, Mischke and Keidel 2003, Gaal et al. 2007, Nielsen et al. 2007).

1.3.6.3 Thromboelastography

Thromboelastography was introduced in the late 1940s (Hartert 1948, 1951). Thromboelastography assesses the changes in viscoelastic properties of whole blood during clot formation under low shear conditions (Kol and Borjesson 2010). The use of whole blood permits assessment of the influence of circulating plasma and cellular components on platelet function and clot formation. Thromboelastography therefore may more accurately reflect *in vivo* haemostasis and more efficiently predict haemostatic kinetics compared to routine plasma-based assays (Hoffman 2001).

Thromboelastography measurements include reaction time, kinetics, maximum amplitude and alpha angle which provide an indicator of the time to initial fibrin formation, speed of clot formation, speed of clot strengthening and clot strength respectively (Jandrey 2012).

1.3.6.4 Platelet activation markers

Platelet activation markers are available and used in humans, infrequently utilised in animals and potentially useful for application to veterinary studies (refer to Section 1.4.1.4). Platelet activation markers may be classified as direct (platelet surface-associated P-selectin expression and BTG and PF4

plasma concentrations) and indirect (soluble P-selectin and thromboxane B2 plasma concentrations).

In addition, as previously discussed (in Section 1.3.4), several of the Advia 120/2120 derived platelet indices, including MPV, MPC concentration and MPM (and their respective distribution widths PDW, PCDW and PMDW) are also (direct) markers of platelet activation.

Platelet surface P-selectin expression

Platelet activation results in degranulation and release of proteins such as P-selectin, which are subsequently transported to the cell membrane. This causes a change in the number of cell surface molecules on circulating platelets, which may be measured by immunofluorescent flow cytometry (Macey et al. 1999, Moritz et al. 2005):

In short, platelets are marked with fluorescence-conjugated antibodies against activation-dependent markers such as P-selectin and passed through a flow cytometer (Zelmanovic and Hetherington 1998). The number of positive events determines the number of activated platelets and the degree of fluorescence relates to the number of activation dependent markers on each platelet (Zelmanovic and Hetherington 1998).

Immunofluorescent flow cytometry is reported as sensitive and specific (Zelmanovic and Hetherington 1998, Ahnadi et al. 2004). However, the sensitivity of the test has been questioned relative to MPC concentration (Moritz et al. 2005). Disadvantages of P-selectin expression assessment include expense and prolonged sample preparation time (approximately 60 minutes) (Zelmanovic and Hetherington 1998).

Soluble P-selectin

Soluble P-selectin is the soluble circulating isoform of P-selectin, which is detectable in blood by enzyme-linked immunosorbent assay (ELISA) methods (Blann et al. 2003). Soluble P-selectin concentration is a less established marker of platelet activation compared to platelet bound P-selectin.

Platelet specific granular proteins i.e. β-thromboglobulin and platelet factor 4

Platelet alpha granules contain a variety of proteins including BTG and PF4 (Kamath et al. 2001). Both BTG and PF4 are specific to platelets, found in similar quantities within alpha granules and are released during platelet activation (Kamath et al. 2001). Therefore increased plasma concentrations are associated with increased *in vivo* platelet activation (Kamath et al. 2001). Detection of BTG and PF4 is achieved using an ELISA or radioimmunoassay (Kamath et al. 2001).

Plasma metabolite thromboxane B2

Plasma thromboxane B2 is the stable, inactive metabolite of plasma thromboxane A2. Plasma thromboxane A2 is generated by platelets from arachidonic acid in response to platelet agonist stimulation (Boudreaux and Catalfamo 2010). Generation of thromboxane A2 causes platelet aggregation and release of dense granule contents (Boudreaux and Catalfamo 2010). Measurement of plasma thromboxane B2 concentrations, via radioimmunoassay or ELISA, are reported methods of assessing platelet activation (Fuse and Kamiya 1994, Tarnow et al. 2005, Nielsen et al. 2007).

Mean platelet component concentration

Refer to Section 1.3.4.3

1.3.7 Platelet evaluation and variation in the Cavalier King Charles spaniel

Cavalier King Charles spaniels have a high prevalence of inherited, subclinical thrombocytopenia and macrothrombocytosis (Eksell et al. 1994, Pedersen et al. 2002, Cowan et al. 2004, Singh and Lamb 2005). These conditions have posed several unique challenges with regard to accurate enumeration of platelet numbers, and evaluation of their function, in this very popular breed. The relevant literature pertaining to these issues is presented in the following pages, starting first with a review of the counting and evaluation methods, and followed by a review of the disorder itself.

1.3.7.1 Platelet evaluation in the Cavalier King Charles spaniel

1.3.7.1.1 Manual methods

Prior to the availability of modern optical haematology instruments, manual methods of platelet counting were preferred for the CKCS breed because of the relative inability of impedance haematology instruments to distinguish large platelets from red blood cells (Brown et al. 1994, Eksell et al. 1994).

One study in CKCS (Singh and Lamb 2005) reported that there was no statistically significant difference in platelet counts obtained by manual counting and blood smear estimation, however Olsen et al. (2004) reported that at lower platelet counts (less than or equal to 100 x 10^9/L), blood smear estimation underestimated platelet counts compared with manual counting, and theorised that this could be due to large platelets localising in the feathered edge of the blood smear. Furthermore, a later study by Tvedten et al. (2008) supported this theory, reporting that CKCS with greater than 50% macrothrombocytes (n=6) and CKCS with less than 50% macrothrombocytes (n=16) had 7.1 times and 2.4 times the number of platelets at the feathered edge of the blood smear than in the monolayer, respectively (Tvedten et al. 2008).

1.3.7.1.2 Automated cell counting

Impedance

The majority of platelet studies in the CKCS breed (n=6) have utilised impedance based instruments due to the unavailability, until recently, of optical based instruments. Five of these studies reported that impedance counters underestimated platelet count compared to manual methods (manual counting or blood smear estimation), due to an inability to consistently distinguish large platelets from erythrocytes (Eksell et al. 1994, Olsen et al. 2001, Olsen et al. 2004, Bertazzolo et al. 2007, Tvedten et al. 2008). The sixth study found no statistically significant difference between manual counting, blood smear estimation or impedance based counting and concluded that automated or blood smear estimates are sufficient to count platelets (Singh and Lamb 2005).

Optical

Five studies have utilised optical haematology analysers in the evaluation of platelet indices in the CKCS.

The first of these assessed one of the earliest optical haematology instruments, the Technicon H1 (Technicon Instruments Corporation, Tarry Town, NY; now Siemens), precursor of the Advia 120 (Bertazzolo et al. 2007). The latter study reported strong correlation between platelet counts obtained by the Technicon H1 and an impedance counter (Hemat 8, SEAC, Florence, Italy) and blood smear estimation. There was however poor agreement between the Technicon H1 and the Hemat 8 and blood smear estimation. This poor agreement was proposed to be due to inaccurate classification of large platelets as erythrocytes (Bertazzolo et al. 2007).

The second study compared optical (Sysmex XT-2000iV PLT-O), impedance (Sysmex PLT-I and Cell-Dyn 3500), manual, and QBC plateletcrit-derived platelet counts (Figure 1.6). When platelet counts were greater than 164 x 10^9/L (i.e. cases not likely to have macrothrombocytosis) all methods (excluding QBC plateletcrit platelet count) provided similar counts. At lower

counts however, the differences between methods were greater. Overall, both optically and manually obtained platelet counts underestimated platelet numbers at lower counts, compared to the QBC plateletcrit, but performed better than impedance methods in these circumstances (Tvedten et al., 2008). The latter study contested the traditional belief that manual counting is more accurate than automated, at least for the Sysmex PLT-O.

Due to copyright restrictions, this figure has been removed.

Figure 1.6: Optical (PLT-O), impedance (PLT-I and CELL-DYN), manual and QBC plateletcrit platelet counts in 27 CKCS.
Figure reproduced from Tvedten et al. (2008). At lower platelet counts, PLT-O and manual counting performed better than PLT-I and Cell-Dyn, consistent with the improved ability of optical haematology instruments (such as the Sysmex XT-2000iV), to discriminate large platelets compared to impedance ones.

The third study to use an optical haematology analyser in the evaluation of platelet indices in the CKCS, reported an association between β1-tubulin mutation and macrothrombocytopenia and used the Advia 120 to obtain platelet counts (Davis et al. 2008) (refer to Section 1.3.7.2.3).

A fourth study assessed the validity of the Advia 2120 generated PCT by comparing it to the QBC derived platelet count (the gold standard for platelet mass, Section 1.3.3.3) (Tvedten et al. 2012). To enable direct study comparisons, units of the Advia 120 PCT (%) were converted to units of the QBC derived platelet count ($x10/L^9$) by multiplication of an estimated

conversion factor (i.e. 1000) (Tvedten et al. 2012). Bland Altman analysis showed fair agreement between the Advia 2120 PCT (x1000) and the QBC derived platelet counts, but the former had a negative bias of 26 x 10^9/L, with underestimation of PCT particularly at lower PCT values (Tvedten et al. 2012). The 95% limits of agreement were -149 to 97 x 10^9/L.

Lastly, a recent CKCS study used both the Advia 120 and 2120 to determine platelet counts and PCT, and concluded that the use of PCT (as determined by the Advia 120/2120), compared to platelet count, avoided overestimation of low platelet mass in CKCS with disease (Kelley et al. 2014).

1.3.7.1.3 Plateletcrit

The PCT, derived from QBC analysis, is considered to be the gold standard for evaluating platelet mass in the CKCS (and other dogs with macrothrombocytosis) (Tvedten et al. 2012). In CKCS, the PCT is typically within reference intervals regardless of platelet count (Bertazzolo et al. 2007, Tvedten et al. 2012). Plateletcrit is therefore of value in these dogs to enable differentiation between inherited macrothrombocytopenia (PCT within reference intervals) and clinically relevant thrombocytopenia (decreased PCT) (Tvedten et al. 2008). A PCT reference interval for CKCS has not been established; however, Tvedten et al. (2012) reported a mean PCT and 95% confidence interval (for 31 clinically healthy CKCS) as 0.27% and 0.1-0.44% respectively (Tvedten et al. 2012).

1.3.7.1.4 Mean platelet volume

In the CKCS there is an inverse relationship between platelet size and platelet count. More specifically dogs with larger platelets have lower platelet counts (Brown et al. 1994, Cowan et al. 2004, Olsen et al. 2004, Singh and Lamb 2005).

One CKCS study assessed MPV generated by the impedance haematology instrument Hemat 8 (SEAC, Florence, Italy) and did not find a significant

difference in CKCS with or without macrothrombocytes (11.6 and 12.1 fL respectively) (Bertazzolo et al. 2007). The same study also assessed the laser cell counter, Technicon H1, and reported that the MPV with or without macrothrombocytes was 5.2 and 6.6 fL respectively, approximately only half that obtained from the impedance instrument. This suggests that both instruments (impedance and older optical) are inaccurate in breeds with macrothrombocytosis due to an inability to detect large platelets (Bertazzolo et al. 2007). However another method to classify the presence or absence of macrothrombocytes was not used.

1.3.7.2 Platelet variation in the Cavalier King Charles spaniel

1.3.7.2.1 Thrombocytopenia

The majority of platelet studies in the CKCS have defined thrombocytopenia as a platelet count less than 100x 10^9/L. This reference interval was originally selected to stringently define dogs with thrombocytopenia (Eksell et al. 1994, Pedersen et al. 2002). Using this definition, the reported prevalences of thrombocytopenia arising from various studies in the CKCS are 32/102 (31%) (Eksell et al. 1994), 9/17 (53%) (Olsen et al. 2001), 59/106 (56%) (Pedersen et al. 2002), 36/69 (51%) (Cowan et al. 2004), 15/43 (35%) (Olsen et al. 2004) and 83/152 (55%) (Singh and Lamb 2005), based on manual platelet counts obtained using a haemocytometer. A further study assessing platelet number with blood smear estimation (as opposed to manual counting) reported a slightly lower prevalence of thrombocytopenia of 9/41 (22%) (Bertazzolo et al. 2007).

Despite these studies, the prevalence of thrombocytopenia in the CKCS, as assessed by a modern optical automated haematology instrument, has not previously been reported. However in two CKCS studies utilising the Sysmex XT-2000iV and Advia 2120, prevalences (using a definition of thrombocytopenia as a platelet count less than 100 x 10^9/L) can be

estimated from the graphs to be (10/27) 37% and (19/43) 44% respectively (Tvedten et al. 2008, Tvedten et al. 2012).

The majority of studies reporting the frequency of thrombocytopenia in the CKCS originate from northern Europe (and in particular Denmark and Sweden); with only one Australian study to date (Singh and Lamb 2005). The latter study defined the lower limit of the reference interval for platelet count as 200 x 10^9/L because this was the limit used by common references (Barger 2003, Boudreaux 2010b) and commercial laboratories, and set by their impedance auto analyser. The latter study reported the frequency of thrombocytopenia as 90% with 25% of cases having severe thrombocytopenia (platelets less than 50 x 10^9/L). If however, the same the lower limit of 100 x 10^9/L was used, the frequency of thrombocytopenia, as reported above, was 55% (Singh and Lamb 2005) which is similar to the prevalence of thrombocytopenia found in CKCS residing in Denmark and Sweden (Pedersen et al., 2002, Eksell et al., 2004., Olsen et al., 2001, 2004).

Thrombocytopenia in the CKCS is typically mild-moderate. The range of the mean and median platelet counts from CKCS platelet studies (Table 1.5) are 87.5-178 x 10^9/L and 92-238 x 10^9/L respectively, with an overall range of 20-489 x 10^9/L.

Count method	Number of dogs	Mean platelet count ($x10^9$/L)	Range ($x10^9$/L)	Standard deviation (SD) ($x10^9$/L)	Median ($x10^9$/L)	Interquar-tile range (IQR) ($x10^9$/L)	95% Confidence interval ($x10^9$/L)	Study origin	Study
Manual count	102	178	NS	104	NS	NS	NS	Sweden	(Eksell et al. 1994)
Manual count	18	124	NS	95	NS	NS	NS	Denmark	(Olsen et al. 2001)
Manual count	105	NS	25-394	NS	92	56-153	NS	Denmark, Sweden	(Pedersen et al. 2002)
Manual count	69	119	20-305	78	108	NS	NS	Tennessee, USA	(Cowan et al. 2004)
Manual count	152	87.5	NS	NS	NS	NS	78.3-97.8	New South Wales, Australia	(Singh and Lamb 2005)
Blood smear estimate	41	NS	NS	NS	238	NS	NS	Italy	(Bertazzolo et al. 2007)
Manual count	27	NS	30-415	NS	NS	NS	NS	Sweden	(Tvedten et al. 2008)
Blood smear estimate	27	NS	26-489	NS	NS	NS	NS	Sweden	(Tvedten et al. 2008)

Table 1.5: Previously reported platelet counts in the CKCS.

1.3.7.2.2 Macrothrombocytosis

A study assessed 14 CKCS with a variety of diseases and found that more than 30% of platelets were larger in diameter (greater than 3.7µm) than platelets from healthy dogs of other breeds (Holme et al. 1981). Brown et al. (1994) subsequently investigated platelet size in 10 healthy CKCS, reporting a significantly larger platelet diameter in the CKCS compared to healthy dogs of other breeds, with median values of 2.5 to 3.75µm and 1.25 to 2.5µm

respectively. Platelet diameter in the CKCS showed bimodal distribution with 44.5% platelets ranging in size from 1.25 to 2.5µm and 30% from 3.75 to 5.0µm (Brown et al. 1994). Both studies used an eyepiece graticle to estimate platelet diameter.

Approximately one-third of CKCS appear to have macrothrombocytosis, as reported in two studies totalling 219 individuals; 46/152 (30%) (Singh and Lamb 2005) and 22/67 (33%) (Cowan et al. 2004).

1.3.7.2.3 Macrothrombocytopenia

Macrothrombocytopenia is defined as macrothrombocytosis and thrombocytopenia occurring together.

The CKCS breed has a high prevalence of macrothrombocytopenia (Pedersen et al. 2002). Several studies suggest that approximately half of all CKCS have platelet variations without bleeding tendencies, characterised by the presence of thrombocytopenia – as assessed by manual count – in 31-56% of dogs (Eksell et al. 1994, Olsen et al. 2001, Pedersen et al. 2002, Cowan et al. 2004, Olsen et al. 2004, Singh and Lamb 2005), and macrothrombocytosis affecting an overlapping 30-33% of dogs (Singh and Lamb 2005).

The majority of CKCS with inherited platelet variation have macrothrombocytopenia (i.e. macrothrombocytosis is seen most often in combination with thrombocytopenia). The first CKCS study to demonstrate this graphically, by plotting MPV against platelet count, was published in 2012 (Tvedten et al. 2012). No automated haematology instrument, prior to the Advia 120 has reportedly produced a reliable MPV in the CKCS. Macrothrombocytosis or thrombocytopenia alone appears to be an uncommon finding.

In one study, 70% of CKCS with macrothrombocytosis had a platelet count less than 100x 10^9/L (Singh and Lamb 2005). Another study in CKCS

reported a significantly lower platelet count (median 63.5 x 10^9/L) in cases with macrothrombocytosis, compared to those with platelet size within reference interval (median 118 x 10^9/L; Mann-Whitney U = 290, p = 0.011) (Cowan et al. 2004). In the latter study however, there was some variability as to whether thrombocytopenia only 20/43 (47%), macrothrombocytes only 8/43 (18%) or both conditions were present 15/43 (35%) (Cowan et al. 2004).

The subclinical nature of macrothrombocytopenia in the CKCS is consistent with adequate platelet function, and is likely reflects the greater functional activity of macrothrombocytes (Smedile et al. 1997).

Macrothrombocytopenia exhibits an autosomal recessive pattern of Mendelian inheritance (Pedersen et al. 2002, Singh and Lamb 2005). Singh and Lamb (2005) demonstrated an equal prevalence in both sexes, generation trait skipping, that parents of affected dogs were often related and that all progeny from two affected parents would be affected. The latter study also reported a high prevalence of affected dogs (90%), consistent with low numbers of non-carriers (Singh and Lamb 2005).

The autosomal recessive nature of macrothrombocytopenia in the CKCS was confirmed by the identification of a mis-sense mutation at coding nucleotide 745 in the gene encoding β1-tubulin (Davis et al. 2008). The mutation results in the substitution of asparagine for aspartic acid at amino acid position 249. The latter forms part of the microtubule intraprotofilament binding site and the net result is impaired microtubule assembly (unstable α-β tubulin dimers within microtubule protofilaments). Impaired microtubule assembly leads to altered proplatelet formation and release by megakaryocytes and results in macrothrombocytopenia (Davis et al. 2008). The lack of bleeding diathesis in macrothrombocytopenic CKCS suggests that the microtubule defect does not alter platelet function significantly (Davis et al. 2008). Additionally, preservation of an adequate platelet mass is likely responsible for maintenance of platelet function in macrothrombocytopenic dogs (Davis et al. 2008).

The prevalence of the mutation is high in the United States with an estimated 92% of CKCS either homozygous or heterozygous for the mutation (Davis et al. 2008). In contrast, in Ireland, 65% of CKCS were reportedly homozygous or heterozygous for the mutation (Davis et al. 2008). Platelet numbers were significantly different among groups based on the presence or absence of the β1-tubulin gene mutation; with low platelet counts (generally less than 100 x 10^9/L), intermediate platelet counts (approximately 200 x 10^9/L) and higher platelet counts (typically greater than 250 x 10^9/L) correlated with a homozygous, heterozygous and clear state for the mutation respectively (Davis et al. 2008). There are currently no reports of the prevalence of the mutation in other parts of the world.

Older studies have reported the β-1 tubulin isotype to be megakaryocyte-specific (Schwer et al. 2001). In contrast, a more recent study which investigated the distribution of β-tubulin isotypes in human tissue reported β-1 tubulin to be relatively ubiquitous, with highest expression in the heart, brain, ovary and thymus (Leandro-García et al. 2010).

1.4 Valvular heart disease and platelet function in people and dogs

In all species, valvular heart disease has the potential to affect platelet activation or function (Goldsmith et al. 2000). Resultant turbulent high-velocity blood flow and fluid shear stresses may decrease platelet function via increased fragmentation of circulating VWF high molecular weight multimers (Tarnow et al. 2004) or increase platelet activation following damage to the vascular endothelium and cellular components of blood (Brown et al. 1975, Stein and Sabbah 1976, Goldsmith et al. 2000). The associations between valvular heart disease and platelet activation or function are not well understood, with conflicting results reported in the human and veterinary literature.

In people, many studies have reported increased platelet activation in heart failure. However, only small numbers have specifically assessed platelet function in people with valvular disease (such as MVP) and many include patients receiving drug therapies with the potential to alter platelet function (Chung and Lip 2006). In addition, it is often unclear if cases are subclinical or clinical, preventing assessment of the association of platelet function with varying severities of heart disease.

Multiple studies in dogs have assessed platelet function exclusively in patients with valvular disease (i.e. CVHD), using numerous methods including aggregometry, PFA-100 testing and thromboelastography; however in contrast to the medical literature, few veterinary studies have used platelet activation markers for this purpose. Unlike their medical counterparts, the majority of canine studies excluded cases receiving drug therapies and included only subclinical CVHD cases (as reviewed by Tanaka and Yamane, 2000).

This section of this thesis will review platelet function in dogs with CVHD as assessed by platelet aggregometry, PFA-100 CT and thromboelastography,

followed by a review of the relatively small number of studies available on platelet activation markers in dogs with CVHD and the comparatively larger numbers of studies reporting platelet activation markers in people with valvular heart disease (including MVP).

1.4.1 Platelet function and associations with chronic valvular heart disease

1.4.1.1 Platelet aggregometry

Most of the published aggregometry-based studies in CKCS have demonstrated an increased platelet aggregation response with varying degrees of severity of subclinical CVHD (regardless of MVR status), suggesting increased platelet reactivity (Olsen et al. 2001, Tarnow et al. 2005, Nielsen et al. 2007). In one of these studies the increased aggregation response was found to be dependent on a platelet count greater than or equal to 100 x 10^9/L (Olsen et al. 2001) (Table 1.6). However, other studies reported decreased platelet aggregation in various dog breeds with clinical CVHD (Tanaka and Yamane 2000) and in CKCS with varying degrees of severity of subclinical CVHD (Cowan et al. 2004). It was suggested that this decreased platelet aggregation could be due to chronic platelet activation and subsequent platelet exhaustion (Tanaka and Yamane 2000) (Table 1.6). The latter authors reported that the concentrations of ADP that produced 50% of the irreversible maximum aggregation (the enhancement of platelet sensitivity concentration) were significantly higher in New York Heart Association (NYHA) class III-IV (n=20) compared to class I-II cases (n=12), consistent with decreased platelet aggregation in more advanced/clinical CVHD cases. However, 9/32 (28%) of dogs were receiving angiotensin converting-enzyme blocker therapy, and the latter has been reported in the human literature to inhibit platelet activation (albeit inconsistently) (Chung and Lip 2006).

Contradictory aggregometry results may reflect differences in the severity of heart disease between the study populations, although the lack of association between platelet aggregation response and CVHD severity in studies by Olsen et al. (2001) and Tarnow et al. (2005) is not highly supportive of this theory. Alternatively the differences may reflect the different methodologies used (optical versus whole blood impedance aggregometry), or represent a lack of standardisation in canine platelet aggregation studies (Cowan et al. 2004, Tarnow et al. 2005).

	Increased platelet aggregation	**Decreased platelet aggregation**	**Platelet aggregation within reference interval**
Conditions	10 CKCS with varying degrees of severity of subclinical CVHD and platelets ≥ 100 x 10^9/L, compared with control dogs and CKCS with platelets < 100x10^9/L (Olsen et al. 2001) 31 CKCS with varying degrees of severity of subclinical CVHD and platelets ≥ 100 x 10^9/L, compared with control dogs (Tarnow et al. 2005). (Dogs with platelets < 100x10^9/L were excluded from the study) 12 CKCS with mild MVP and regurgitant jet size ≤ 20% (Nielsen et al. 2007) compared to control dogs. (Dogs with regurgitant jet size > 20% were excluded from the study).	69 CKCS with varying degrees of severity of subclinical CVHD, compared to control dogs and regardless of platelet count (Cowan et al. 2004) 32 dogs (various breeds) with symptomatic CVHD (New York Heart Association class III or IV cases), compared to control dogs (Tanaka and Yamane 2000)	9 CKCS with varying degrees of severity of subclinical CVHD and platelets < 100 x 10^9/L (Olsen et al. 2001)

Table 1.6: Summary of the results of platelet aggregation studies in dogs with CVHD.

1.4.1.2 Platelet function analyser closure times

Platelet function analyser (PFA-100)-based studies have consistently reported increased CT in CKCS with mild-marked subclinical CVHD

suggesting decreased platelet function in such cases (Tarnow et al. 2003, Tarnow et al. 2005) (Tables 1.7 & 1.8 respectively). However, unlike aggregometry-based tests, CT was affected by quantitative and qualitative changes in circulating VWF (Tarnow et al. 2005).

Continuous high shear stress from turbulent blood flow (associated with valvular regurgitation) results in increased fragmentation of circulating VWF high molecular weight multimers due to proteolysis of VWF (by the plasma metalloprotease, ADAMTS13, that cleaves VWF under high shear stress) (Tarnow et al. 2004). Therefore, the described increase in closure time likely reflects an alteration in VWF rather than a change in intrinsic platelet function (Tarnow et al. 2005). A decrease in VWF high molecular multimers is likely to be inversely proportional to increasing shear force because of the positive association between closure time and the degree of valvular regurgitation (Tarnow et al. 2003). Platelet aggregation at high shear rates is reliant on circulating VWF (Tarnow et al. 2005). Therefore, *in vitro* platelet function assays based on physiologic high shear rates and adhesion to a contact surface (e.g. PFA-100) will detect platelet dysfunction if a qualitative VWF defect is present; whereas low shear tests such as platelet aggregometry will be unaffected (Tarnow et al. 2005).

The form of turbulent blood flow does not appear to be an important factor in platelet hypofunction (as assessed by CT), nor is it thought to be breed specific (Clancey et al. 2009a). Other studies have demonstrated increased closure times in a variety of dog breeds with various cardiac diseases including subaortic stenosis (Tarnow et al. 2005, Clancey et al. 2009a, Moesgaard et al. 2009).

	Control mixed breed Absent or minimal MVR (n=15) (Jet size ≤ 10%)	Control CKCS Absent or minimal MVR (n=53) (Jet size ≤ 15%)	Mild MVR (n=15) (Jet size > 15 ≤ 50%)	Moderate-severe MVR (n=18) (Jet size > 50%)
Closure time (seconds)	62 (55-66)	55 (52-64)	75 (60-84) *	87 (66-102) **

Table 1.7: Closure times (using collagen/adenosine diphosphate cartridges) with varying degrees of subclinical mitral regurgitation as assessed by the mitral regurgitant jet size (From Tarnow et al., 2003).
Data reported as median and 25th-75th percentiles. Results are different from the control CKCS at * $p < 0.01$ and ** $p < 0.0001$.

	Control mixed breed Absent or minimal MVR (n=14) (Jet size ≤ 10%)	Control CKCS Absent or minimal MVR (n=14) (Jet size ≤ 20%)	Moderate-severe MVR (n=17) (Jet size ≥ 50%)
Closure time (seconds)	62 (58-68)	64 (57-84)	106 (94-114) **

Table 1.8: Closure times (using collagen/adenosine diphosphate cartridges) with varying degrees of subclinical mitral regurgitation as assessed by the mitral regurgitant jet size (From Tarnow et al., 2005).
Data reported as median and 25th-75th percentiles. Results are different from the control CKCS and control mixed breed dogs at ** $p < 0.0001$.

1.4.1.3 Thromboelastography

Only a single study has used thromboelastography to assess platelet function in CKCS with varying degrees of severity of subclinical CVHD (Tarnow et al., 2010); out of 25 dogs, 11 had absent or minimal MVR, and 14 had severe MVR (regurgitant jet size greater than of equal to 50%). A control group of 8 dogs was also included. These authors reported there were no

statistically significant differences in thromboelastography variables (R, K, MA and α) between the groups, results that did not support global hypercoagulability in CKCS with subclinical CVHD (Tarnow et al. 2010b).

1.4.1.4 Platelet activation markers

Platelet surface P-selectin expression

Martini et al. (1996) investigated platelet surface associated P-selectin expression in people with varying degrees of severity of MVP. The latter study investigated 39 people with absent or mild to moderate MVR, 15 people with severe MVR and 5 healthy controls and found no difference in P-selectin expression between groups (Martini et al. 1996). It was not specified if the cases were subclinical or clinical and therefore it is unclear whether they were congestive heart failure cases or heart disease cases alone.

In contrast, Chung et al. (2007) reported higher P-selectin surface expression by flow cytometry, consistent with increased platelet activation, in people with acute decompensated congestive heart failure due to impaired left ventricular systolic function (22 NYHA class III-IV patients), compared to people with stable congestive heart due to impaired left ventricular systolic function (53 NYHA class I-II and15 class III-IV patients) and healthy controls (n=23). Due to the fact, however, that the number of valvular cases in the latter study was not published, an association between decompensated valvular disease and increased platelet activation cannot be ascertained. To be included in the stable congestive heart failure group there had to be no hospital admission for greater than or equal to 3 months.

A single study in dogs has shown no significant difference in platelet surface associated P-selectin expression, assessed by flow cytometry, in 31 CKCS with either subclinical CVHD or subaortic stenosis compared to control dogs, suggesting that platelets do not circulate in a pre-activated form in either disease (Tarnow et al. 2005).

Soluble P-selectin

In people, multiple studies have reported a significant increase in soluble P-selectin concentration in congestive heart failure compared to controls (O'Connor et al. 1999, Gibbs et al. 2001, Yin et al. 2003), including one study that included a high proportion (30/74) of valvular heart disease cases (Yin et al. 2003). In addition, soluble P-selectin concentrations increased with congestive heart failure severity in the latter study.

Soluble P-selectin concentration, as an indicator of platelet activation, has not been investigated in dogs with any form of heart disease.

Platelet specific granular proteins i.e. β-thromboglobulin and platelet factor 4

The concentrations of platelet specific proteins released from alpha granules, in particular BTG and PF4, have been used to assess platelet activation in people with MVP and/or MVR with conflicting results. Approximately half of all these studies report BTG and/or PF4 concentrations consistent with *in vivo* platelet activation (Cudillo et al. 1983, Fisher et al. 1983, Arocha et al. 1985, Tse et al. 1997). By contrast, the other half of the literature reports no significant difference in BTG or PF4 concentrations between MVP patients and controls (Scharf et al. 1982, Lin et al. 1989, Martini et al. 1996).

Commercial antibodies against canine BTG are currently unavailable therefore no studies have been performed in dogs (Christopherson et al. 2012).

Plasma metabolite thromboxane B2

To the author's knowledge, no studies have studied thromboxane B2 concentrations in valvular disease in people. A single study reported no significant difference in plasma thromboxane B2 concentration assessed by ELISA, in CKCS with varying degrees of severity of subclinical CVHD compared to a control population (Tarnow et al. 2005).

Mean platelet component concentration

Chung et al. (2007) reported a decrease in MPC concentration, consistent with increased platelet activation, in people with stable congestive heart failure due to impaired left ventricular systolic function (53 NYHA class I-II and 15 class III-IV patients) compared to healthy controls (n=23) but not in acute decompensated congestive heart failure cases (22 NYHA class III-IV patients) compared to controls. However, as the number of MVP cases was not reported, an association between valvular disease and increased platelet activation could not be ascertained. MPC concentration, as an indicator of platelet activation, has not been investigated in dogs with any form of heart disease (including valvular disease).

1.4.2 Significance of increased platelet activation/function in dogs with chronic valvular heart disease

From the foregoing review, it is apparent that the nature and extent of platelet dysfunction associated with valvular disease in dogs is not completely clear. In addition, the consequence(s) of potentially altered platelet function in dogs with CVHD is unclear. It is theorised that activated platelets may contribute to the development of vascular changes such as arteriosclerosis of intra-myocardial coronary arteries and myocardial microthrombosis (Patterson et al. 1961, Jönsson 1972, Whitney 1976, Tarnow et al. 2005). One study reported that these changes were accompanied by focal myocardial necrosis/fibrosis (Patterson et al. 1961).

This could result in decreased myocardial oxygenation (and progression of heart disease) (Falk and Jönsson 2000). Studies in both dogs and people have reported an association between intra-myocardial coronary arteriosclerosis and risk of sudden death (Burke and Virmani 1998, Falk and Jönsson 2000).

More recently, a study reported that 21 dogs with congestive heart failure due to CVHD had significantly advanced intra-myocardial arteriosclerosis and myocardial fibrosis compared to 21 age-matched control dogs euthanased for reasons not associated with cardiac disease (Falk et al. 2006). A direct association between the degree of arterial change and clinical signs of CHVD has also been demonstrated (Falk et al. 2007).

Arterial pathology is common in the CKCS breed (Buchanan et al. 1997, Karlstam et al. 2000). Karlstam et al. (2000) reported pulmonary artery lesions (including moderate to severe intimal thickening) associated with moderate to severe CVHD in seven consecutive CKCS post mortem examinations. Additionally, another CKCS study reported the presence of intimal thickening and breaks in the internal elastic lamina of the femoral artery with associated thrombosis and vascular occlusion (Buchanan et al. 1997).

In conclusion, a direct link between arterial pathology and CVHD in dogs has not been determined; however, both conditions commonly coexist. It has been theorised that activated platelets could contribute to the development of vascular changes and myocardial microthrombi and subsequently, heart disease progression, and that narrowing of intra-myocardial arteries due to coronary arteriosclerosis could produce high shear stresses which in turn could activate platelets (Tarnow et al. 2005).

2 Project Aims and Hypotheses

As noted in the literature review, few studies have assessed platelet activation markers in CVHD in dogs. Therefore, the present study sought:

1. To determine the frequency of CVHD in a population of CKCS in Western Australia.

2. To characterise and compare platelet indices in the above CKCS

- To assess automated platelet indices in the CKCS.
    - Due to the inherent variation in platelet number and size in the CKCS, the assessment of individual platelet indices in this breed is of interest. The exploration of individual platelet indices (such as PCT) and the association between platelet indices (such as MPV and platelet count) forms the basis for the first aim of this present study.
- To assess the agreement between two platelet counting methods (blood smear estimate and optical count) in CKCS with macrothrombocytopenia.
    - Despite improved accuracy over impedance counters, the Advia 120 may have some difficulty differentiating large platelets from erythrocytes at lower counts (Tvedten et al. 2012). Therefore a further aim was to assess the agreement between platelet counting methods (Advia 120 and blood smear estimation). We hypothesise that despite improved accuracy over impedance counters, the Advia 120 may still underestimate platelet counts in cases of macrothrombocytosis (Tvedten et al. 2012).
- To assess the prevalence of macrothrombocytopenia in a population of CKCS in Western Australia.
    - The prevalence of macrothrombocytopenia has not previously been reported in Western Australia. We hypothesise that Western Australian CKCS have a high prevalence of

macrothrombocytopenia as previously reported in another Australian state and overseas.

- To assess the validity of the Advia 120 APCC for the detection of platelet clumps in dogs.
    - No previous study has reported on the APCC in clinically well dogs. Therefore a minor aim was to assess the validity of the Advia 120 APCC for detecting platelet clumps in dogs.

3. To document platelet activation and function and in dogs with subclinical CVHD.

- Significantly longer closure times have been described in CKCS with moderate to severe MVR than for those with minimal or mild regurgitation or healthy control dogs (Tarnow et al. 2003, Tarnow et al. 2004, Tarnow et al. 2005). This finding is due to quantitative and qualitative changes in VWF, rather than an alteration of intrinsic platelet function. Concurrent alteration of platelet function or platelet activation however cannot be excluded. A relatively novel marker of platelet activation, MPC concentration, has been reported in dogs (Moritz et al. 2003, Moritz et al. 2005, Bauer et al. 2012) but the change in MPC concentrations in CVHD is unknown. A 2005 study by Moritz et al., suggested that MPC concentration may be more sensitive than P-selectin at detecting platelet activation. From MPC concentration, the PCDW, an indicator of the variation in platelet density is derived. PCDW is high if both non-degranulated and degranulated circulating platelets are present. An increased PCDW is therefore also a marker of platelet activation (Moritz et al. 2005, Boudreaux 2010b).

4. To determine the factors associated with alterations in markers of platelet activation and function in dogs with subclinical CVHD.

- We hypothesise that as heart disease advances, MPC concentration will decrease (representing platelet activation), and PCDW will increase (representing a variation in platelet density) and that closure

time will increase with advancing heart disease severity (representing decreased platelet function). Additional confounding factors, such as platelet count and HCT, will also be explored.

3 Materials and methods

3.1 Animals

Cavalier King Charles Spaniels

Client-owned CKCS were prospectively recruited from the Murdoch University Veterinary Hospital (MUVH), primary care veterinary practices, and CKCS breeders. Recruitment was sought via distribution of recruitment flyers to CKCS owners who attended MUVH, posting of a recruitment flyer, in the March 2012 edition of the MUVH's bulletin, posting of a recruitment flyer in the April 2012 Western Australian Division of the Australian Veterinary Association's Bulletin, phone contact of CKCS breeders who had previously attended MUVH for heart scoring, email contact with referring veterinarians and informal word-of-mouth recruitment.

Dogs were recruited from September 2011 through June 2013. Dogs were eligible for inclusion if they were greater than or equal to 6 months old and apparently healthy (defined as the absence of systemic or organ-related disease, other than the presence of a left apical systolic murmur or dental disease) as determined by an interview with the owner and results of a health questionnaire and physical examination. Dogs that received any drug (other than a routine prophylactic antiparasitic drug or vaccination) within 8 weeks prior to participation were excluded. Age, sex, and body weight of each dog were recorded.

Control dogs

A control group of healthy dogs of breeds other than CKCS was included for comparison of platelet indices. Client-owned dogs that were not of CKCS breed were retrospectively recruited on the basis of review of the MUVH database for the period of November 2011 through May 2013. Dogs were included if they were greater than or equal to 6 months old, were apparently healthy (defined as the absence of systemic or organ-related disease, other than the presence of dental disease, cutaneous masses less than 1 cm

diameter that appeared non-painful, or orthopaedic disease), and had haematologic analysis performed by use of the Advia 120. Dogs that received any drug (other than routine prophylactic antiparasitic drugs or vaccinations) within 8 weeks prior to participation or had a heart murmur or incomplete medical records were excluded. The reason for admission, signalment (age, sex, and breed), body weight, platelet count, MPC concentration, PCDW, MPV, PDW, PCT, large platelet index and HCT were recorded.

Signed owner consent was obtained for all CKCS participating in the study. The study was approved by the Murdoch University Animal Ethics Committee (R2443/11).

3.2 Clinical heart evaluation

3.2.1 Murmur intensity/grade

Cardiac auscultation of each CKCS was performed prior to echocardiographic assessment by a Diplomate of the European College of Veterinary Internal Medicine - Companion Animals (Internal Medicine) or Fellow of the Australian and New Zealand College of Veterinary Scientists in Small Animal Internal Medicine. The presence or absence, intensity (grades I through VI) (Table 3.9), location, and character of each murmur were recorded (Tilley et al. 2006).

Murmur grade	**Murmur intensity**
I	Very soft
II	Soft
III	Moderate (as loud as the heart sounds S1, S2)
IV	Loud
V	Loud with precordial thrill
VI	Very loud

Table 3.9: Cardiac murmur intensity and grading system.

3.3 Echocardiography

Echocardiography was performed with an ultrasonography system (ACUSON Sequoia^{TM} 512, Siemens, San Jose, CA). Each CKCS was examined while positioned in right and left lateral recumbency. No chemical restraint was used. All examinations were performed by a single investigator, an Internal Medicine Resident (Linda Tong), and all recorded images were reviewed by a Diplomate of the European College of Veterinary Internal Medicine - Companion Animals (Cardiology) (Anne French). Examinations were performed both with and without colour flow mapping by use of a 4- to 10-Hz and 1- to 4-Hz electronic sector transducer, respectively.

3.3.1 Echocardiographic evaluation of left heart remodelling

The LVDD and LVDS were measured by use of M-mode echocardiography at the level of the chordae tendinae as guided by a 2-dimensional right parasternal short axis view (Thomas et al. 1993). LVDD and LVDS were measured when the left ventricle was at its largest and smallest diameter respectively. Measurements were made using a leading edge methodology (from the anterior-most edge of endocardial lines); to include the proximal but not the distal endocardium for each measurement (Sahn et al. 1978). A simultaneous ECG was not recorded during echocardiography. The reported measurement (in cm) for each dog was the mean of 3 measurements normalised on the basis of body weight by use of the following equations (Cornell et al. 2004):

$$LVDDN = LVDD/(\text{body weight in kg})^{0.294}$$

$$LVDSN = LVDS/(\text{body weight in kg})^{0.315}$$

Reference intervals for LVDDN and LVDSN were 1.27 to 1.85 and 0.71 to 1.26, respectively (Cornell et al. 2004).

Left atrial and aortic root diameters were measured by use of a 2-dimensional right parasternal short-axis view at the level of the aortic valve (Thomas et al. 1993) when the left atrium was subjectively, at its maximal

diameter. These diameter measurements were used to calculate LA:Ao. The reported LA:Ao was the mean of 3 ratios calculated from 3 paired measurements. The criterion for left atrial enlargement was LA:Ao greater than 1.5.

Cardiac remodelling was defined as the presence of left atrial enlargement (LA:Ao greater than 1.5), left ventricular enlargement (LVDDN or LVDSN above reference interval), or both (Hansson et al. 2002, Häggström et al. 2008).

3.3.2 Echocardiographic assessment of mitral valve regurgitation

Presence of MVR and estimation of the severity was made with colour flow Doppler echocardiography by use of the left apical 4-chamber view with the dogs positioned in left lateral recumbency (Thomas et al. 1993). Images were analysed frame by frame to estimate the percentage of the left atrium occupied by the largest mitral jet (jet size) as previously reported (Tarnow et al. 2003). Mitral valve regurgitation was subsequently classified as absent to minimal (jet size < 15%), mild (jet size 15% to 50%), or moderate to severe (jet size greater than 50%), as reported elsewhere (Tarnow et al. 2003, Tarnow et al. 2004).

3.3.3 Canine chronic valvular heart disease score

Based upon clinical and echocardiographic findings, CKCS were grouped according to the ACVIM canine CVHD scoring system (Atkins et al. 2009) (Table 3.10).

ACVIM canine CVHD score	Criteria
A	No identifiable structural disorder of the heart, but high risk for developing heart disease (eg. every Cavalier King Charles Spaniel without a heart murmur)
B1	Asymptomatic patients that have no radiographic or echocardiographic evidence of cardiac remodelling
B2	Asymptomatic patients that have haemodynamically significant valve regurgitation, as evidenced by radiographic or echocardiographic findings of left-sided heart enlargement
C	Past or current clinical signs of heart failure associated with structural heart disease
D	End-stage disease with clinical signs of heart failure caused by CVHD that are refractory to standard therapy

Table 3.10: The American College of Veterinary Internal Medicine canine chronic valvular heart disease scoring system.

3.4 Platelet haematology

3.4.1 Blood collection and handling

A blood sample (10 mL) was collected from each CKCS via jugular venipuncture with a 21-gauge needle and 10-mL syringe. Two millilitres of blood were transferred into a tube containing K3EDTA (Vacuette, Greiner Bio-One GmbH, Kremsmünster, Austria), which was used for automated haematologic and blood film assessment. Four millilitres were placed into two 2-mL tubes containing 3.8% sodium citrate (Sarstedt, Nümbrecht, Germany), which were used for closure time assessment. The remaining 4 mL of blood was stored for use in another study. All tubes were filled to the manufacturer's specified volume. Blood was mixed with the anticoagulant by gentle inversion of tubes immediately after sample collection. Blood tubes were mechanically rotated with a blood tube rotator (Innovative Medical Systems Corp, Ivyland, PA.) until processing. Blood was stored at room temperature (approximately 25°C).

3.4.2 Assessment of platelet number and function

Blood samples were analysed using the Advia 120 haematology system and manufacturer-developed multispecies software (version 3.1.8.0-MS, Siemens Healthcare Diagnostics, Tarrytown, NY). All samples were processed within 40 minutes after collection. Platelet count, MPC concentration, PCDW, MPV, PDW, PCT, MPM, PMDW, large platelet index, APCC and HCT were recorded.

Assessment of macrothrombocytosis was also made by dividing the number of large platelets (reported as a number x 10^9/L) by the total Advia 120 platelet count and multiplying this number by 100. If the result was greater than 30%, then the dog was considered to have macrothrombocytosis.

Closure time was determined by use of the PFA-100 analyser and collagen-ADP cartridges in accordance with the manufacturer's instructions. All samples were processed within 60 minutes after collection. Each sample was assayed in duplicate. When the coefficient of variation was greater than 15%, the results were rejected, and a duplicate assay was repeated. Results for dogs with a HCT < 0.35L/L were excluded (Clancey et al. 2009b).

3.4.3 Assessment of blood smears

Blood films were prepared from each CKCS sample within 60 minutes of sample collection and stained with modified Wright stain (Hematek Stain Pak, Siemens, Healthcare Diagnostics Inc., Tarrytown, NY, USA) by use of an automated staining instrument (Hematek 1000, Siemens Healthcare Diagnostics).

All microscopic examinations were performed under 100-1000X magnification, as detailed below. A 10X ocular lens was used in all examinations. One investigator (Linda Tong), who was not aware of the platelet count, examined each blood film. Blood smear platelet estimates

were determined by counting the number of platelets in 10 consecutive oil immersion (100X) objective fields in the monolayer area of each blood smear. The average number of platelets per field was multiplied by 15 x 10^9/L to obtain the platelet estimate as previously described (Tvedten et al. 2008).

The percentage of macrothrombocytes was determined by counting the number of platelets that were subjectively as large as or larger than a red blood cell, in the same 10 consecutive oil immersion (100X) objective fields used for blood smear estimation (Figure 3.7). The number of macrothrombocytes counted was divided by the total number of platelets in those fields to provide a macrothrombocyte percentage. If more than 30% of the platelets were macrothrombocytes, a diagnosis of macrothrombocytosis was made; as previously reported (Brown et al. 1994, Singh and Lamb 2005).

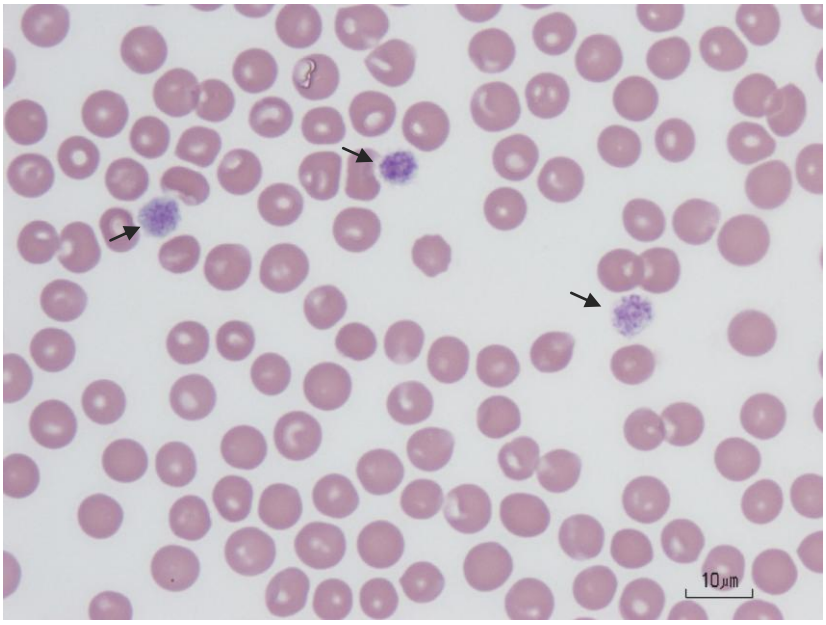

Figure 3.7: Blood smear from a 3-year-old CKCS demonstrating macrothrombocytes
(platelets subjectively assessed to be as large as or larger than a red blood cell) (arrows).

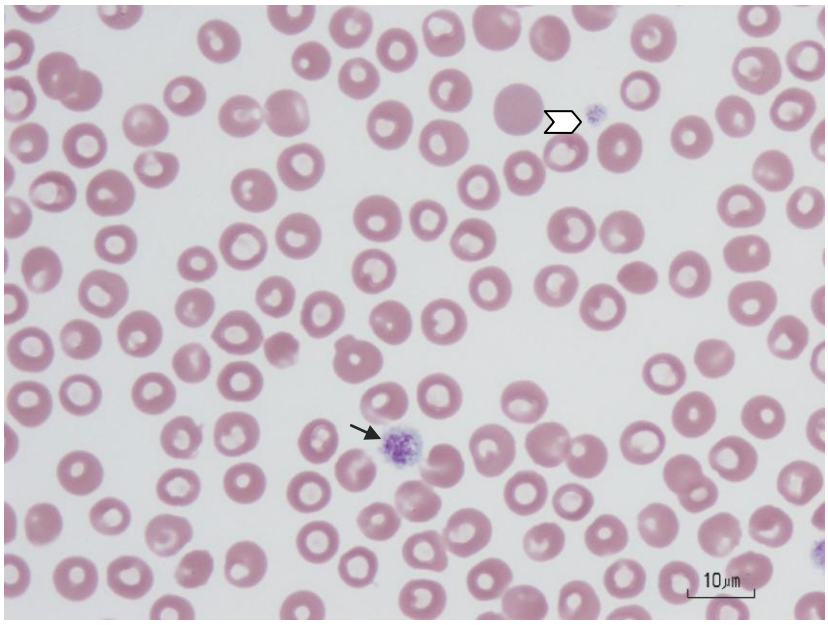

Figure 3.8: Blood smear from a 3-year-old CKCS demonstrating platelet size variation.
Normal sized platelet (arrow head) and macrothrombocyte (arrow).

The number and size of platelet aggregates were estimated in 5 consecutive high-power fields (hpf) (400X magnification) at the feathered edge of each blood film. This protocol was a modification of that described in a study (Bertazzolo et al. 2007) in which the number and size of platelet aggregates were estimated in fields by use of oil immersion (1000X magnification). The number of platelet aggregates was judged on a scale of 0 to 3 as follows: 0 = absent, 1 = sparse with 1 or 2 aggregates/5 hpfs, 2 = moderately frequent with 3 to 5 aggregates/5 hpfs, and 3 = numerous with greater than 5 aggregates/5 hpfs. The size of aggregates was assessed on a scale of 1 to 3 as follows: 1 = small (3 to 8 platelets), 2 = medium (9 to 20 platelets), and 3 = large (greater than 20 platelets).

3.5 Statistical analysis

Descriptive variables with continuous data (age, body weight, platelet count, MPC concentration, PCDW, and HCT) were tested for normality by use of the Shapiro-Wilk test. A normal distribution was determined when there was failure to reject the null hypothesis of normality at $P < 0.05$. Normally distributed data were summarised as mean and 95% CI. Non-parametric data were summarised as median and IQR. Comparisons were made between groups by use of Student *t* tests for parametric variables and the Mann-Whitney *U* test for nonparametric variables. Estimated frequencies of select variables were summarised as proportion and 95% CI. The distribution of sex for each group was compared by use of a Fisher exact test. Comparisons among 3 or more categories within CKCS cohorts were performed by use of an analysis of variance (ANOVA) for parametric responses or a Kruskall-Wallis test for nonparametric responses. Pairwise *post hoc* comparisons were performed by use of the least squares mean or the Mann-Whitney *U* test, respectively, with a Bonferonni correction for overall type I error. Significance was set at $P < 0.05$ for all comparisons.

Measured variables for the CKCS group were summarised as described previously. Associations between possible explanatory variables (sex, age, murmur, jet size, LA:Ao, LVDDN, LVDSN, platelet count, and HCT) and outcomes (closure time, MPC concentration, and PCDW) were assessed with multivariate regression models using SAS version 9.4 (SAS Institute, NC, USA). The model of best fit was selected on the basis of the Cp statistic and consideration of the R^2. All possible subsets were evaluated. The best models for subsets were selected on the basis of the smallest Cp statistic with the least bias (i.e., the Cp statistic closest to p, where p is the number of variables in the model). The simplest subsets that explained the outcome were chosen with the selected Cp statistic and R^2. The proportion of variance explained by the explanatory variable or variables, partial R^2, and corresponding *P* value were reported.

The level of agreement between platelet-counting methods (blood smear estimation and the Advia 120) was assessed using Bland-Altman analysis.

A comparison between automated platelet clump flag and visual detection of platelet clumping was calculated using Kappa statistics, with GraphPad® software on-line calculator (http://graphpad.com/quickcalcs/kappa1.cfm).

The confidence intervals of proportions were calculated with an on-line calculator (http://vassarstats.net/prop1.html).

4 Results

4.1 Animals

A total of 89 CKCS fulfilled the inclusion criteria; 47 were female (34 spayed and 13 entire) and 42 male (38 castrated and 4 entire). In the control group, 39 dogs fulfilled the inclusion criteria; 20 were female (17 spayed and 3 entire) and 19 male (16 castrated and 3 entire). The breeds in the control group are detailed in Table 4.11.

Breeds	**Number of dogs**
Cross breed	15
Greyhound	4
Poodle	3
Labrador retriever	2
Maltese	2
Jack Russell terrier	2
Staffordshire bull terrier	2
Pekingese	1
Dachshund	1
Blue heeler	1
Cocker spaniel	1
Schnauzer	1
Australian cattle dog	1
German shorthaired pointer	1
German shepherd dog	1
Shih Tzu	1

Table 4.11: Dog breeds of the control group (n=39).

There was no significant difference in the frequency of sex between groups (P = 0.150; Fisher exact test).

Ages and bodyweights of the CKCS and control dogs are summarised in Tables 4.12 and 4.13 respectively. There were significant differences between both age and bodyweight of CKCS and control dogs ($P < 0.001$ and $P = 0.01$, respectively; Mann Whitney).

Age (years)	CKCS	Control dogs
Range	0.6-15	0.8-15
Median	4	9.8
Interquartile range (IQR)	2-6	5.5-12.3

Table 4.12: Ages of the CKCS (n=89) and control dogs (n=39).

Bodyweight (kg)	CKCS	Control dogs
Range	5-18	3-35
Median	10.1	20.5
IQR	8.2-11.6	6.4-29

Table 4.13: Bodyweight of the CKCS (n=89) and control dogs (n=39).

4.2 Heart disease

4.2.1 Murmur grade

A left apical systolic murmur was detected in 51 of 89 CKCS (57%; 95% CI, 46% to 66%). Of the 51 CKCS with a murmur, 24 (47%) were aged 6 years or less. The frequencies of the murmur grades are listed in Table 4.14 and the relationship between age and murmur grade is displayed in Figure 4.9. There was a positive relationship between murmur grade and age.

Murmur grade	Number of CKCS
0	38
I	20
II	13
III	9
IV	7
V	2
VI	0

Table 4.14: Frequency of murmur grades in the CKCS (n=89).

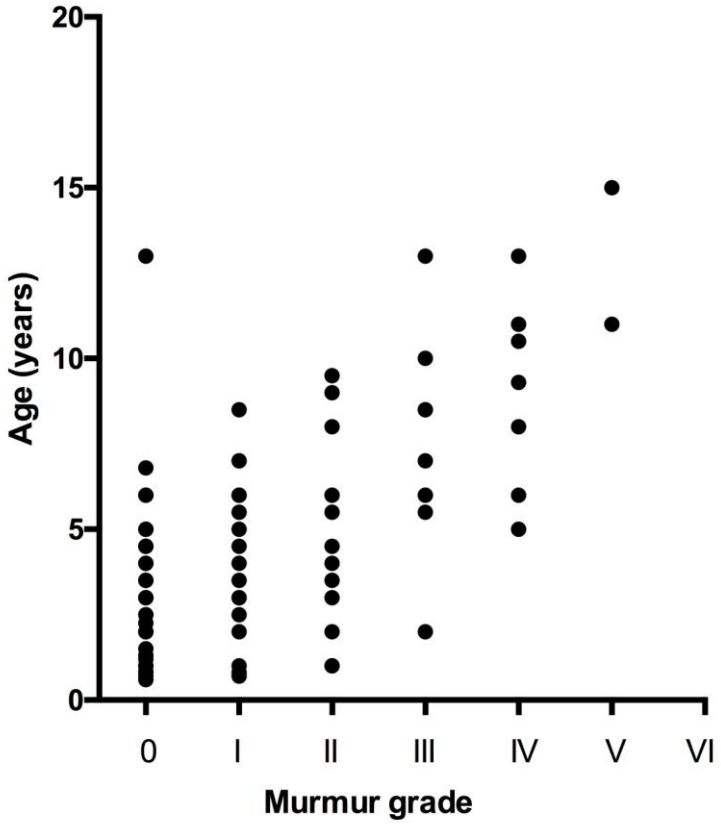

Figure 4.9: Murmur grade and age of each CKCS (n=89).

4.2.2 Regurgitant jet size

Mitral valve regurgitation was detected by use of colour-flow Doppler echocardiography in 86 of 89 (97%; 95% CI, 90% to 99%) CKCS. The 3 dogs that had no evidence of MVR were 8 months old, 3 years old, and 4 years old).

The number of dogs within each regurgitant jet size group is detailed in Table 4.15.

Severity group	Regurgitant jet size (%)	n
Absent-minimal	< 15	52
Mild	15 to 50	14
Moderate-severe	> 50	23

Table 4.15: Regurgitant jet size severity group for 89 CKCS with subclinical CVHD.

4.2.3 Echocardiographic indices of left heart remodelling

The results of echocardiographic indices of cardiac remodelling (LA:Ao, LVDDN and LVDSN) are shown in Table 4.16 and Figure 4.10.

Left atrial enlargement was evident in 22 of 89 CKCS. Four of 89 CKCS had left ventricular enlargement during diastole. Left ventricular enlargement during systole was not detected in any CKCS.

	LA:Ao	LVDDN	LVDSN
Range	1-2.08	0.95-2.06	0.51-1.26
Mean	1.40	1.40	0.86
95% CI (mean)	1.35-1.45	1.35-1.44	0.83-0.89

Table 4.16: Mean LA:Ao, LVDDN and LVDSN values for the CKCS (n=89).

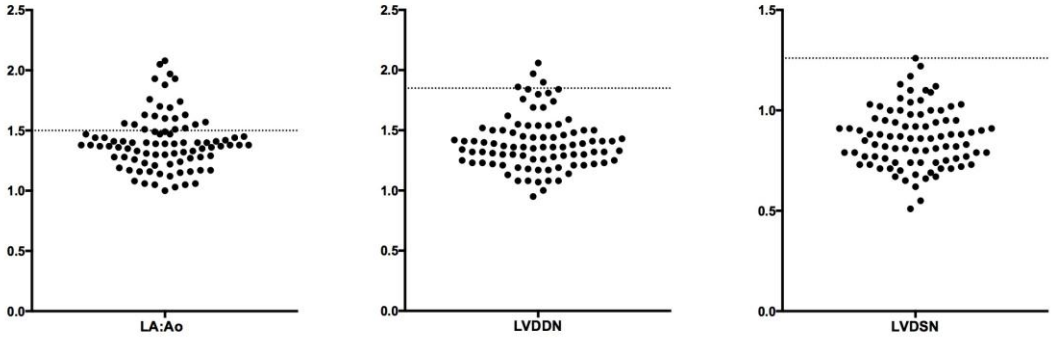

Figure 4.10: LA:Ao, LVDDN and LVDSN values for each CKCS (n=89).

In each graph, the dotted line indicates the upper limit of the reference interval.

4.2.4 Canine chronic valvular heart score

The ACVIM canine CVHD scores are summarised below (Table 4.17). As only subclinical cases were included in the present study, no dogs were classified in Stage C or D

ACVIM canine CVHD Stage	Number of CKCS
A	3
B1	73
B2	13
C	0
D	0

Table 4.17: The ACVIM canine CVHD scores of the CKCS (n=89).

4.3 Platelet enumeration

Cavalier King Charles spaniel Advia 120 and blood smear derived platelet counts and control dog Advia 120-derived platelet counts are summarised in Table 4.18 and displayed in Figure 4.11. There was a significant difference between the Advia 120-derived platelet counts in the CKCS and control dogs ($P < 0.001$; Mann Whitney). Thrombocytopenia, defined as an Advia 120-derived platelet count less than 100 x 10^9/L, was identified in 18 of the 89 (20%; 95% CI, 13% to 30%) CKCS but was not identified in any of the control dogs.

Platelet count (x 10^9/L)	**CKCS**		**Control dogs**
	Advia 120	**Blood smear estimate**	**Advia 120**
Range	27-565	33-642	123-699
Median	213	220.5	315
IQR	143-306	132-296	171-402

Table 4.18: Platelet counts (obtained by the Advia 120 and blood smear estimation) in the CKCS (n=89) and (by the Advia 120) in the control dogs (n=39).

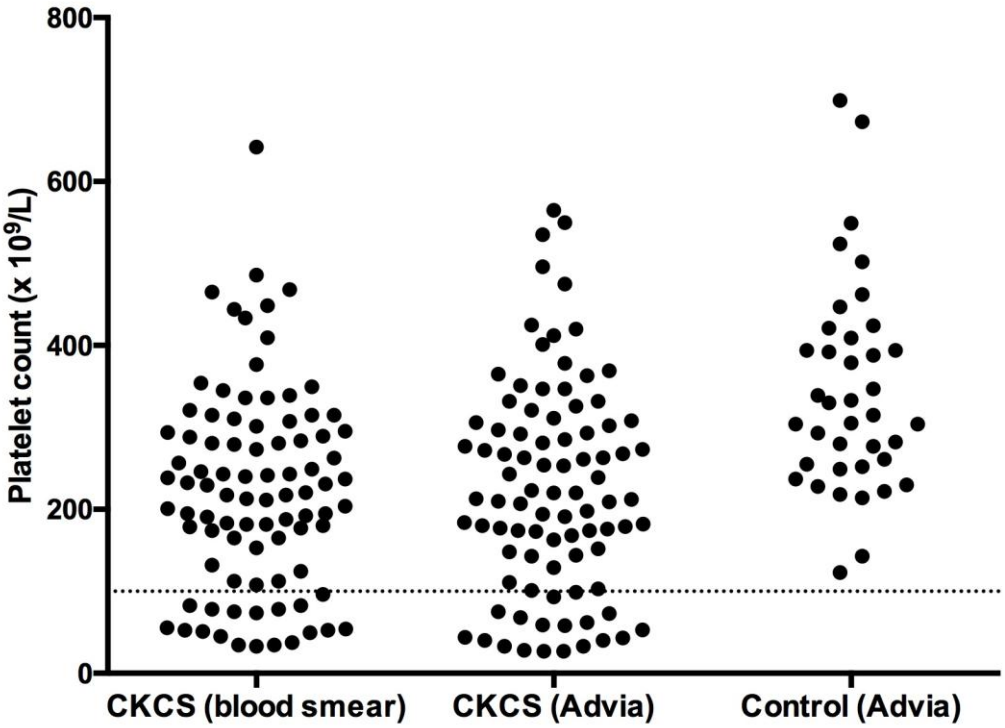

Figure 4.11: Platelet counts of the CKCS (obtained by blood smear estimation and the Advia 120; n= 89) and control dogs (obtained by the Advia 120; n=39).
Both control dogs with a platelet count of < 200 x 10^9/L were Greyhounds. The dotted line indicates a platelet count of 100 x 10^9/L.

Analysis of the agreement between platelet counting obtained by smear estimation and the Advia 120 was made using Bland–Altman agreement analysis (Figure 4.12).

The plot of the difference between smear estimation and Advia 120 values against their means according to the Bland and Altman design, showed a bias of -1.82 x 10^9/L, with a SD of 38.58, and with 92.1% of differences within the agreement limits (mean ± 2SD). This confirmed good agreement between platelet counting methods with clinically insignificant variation.

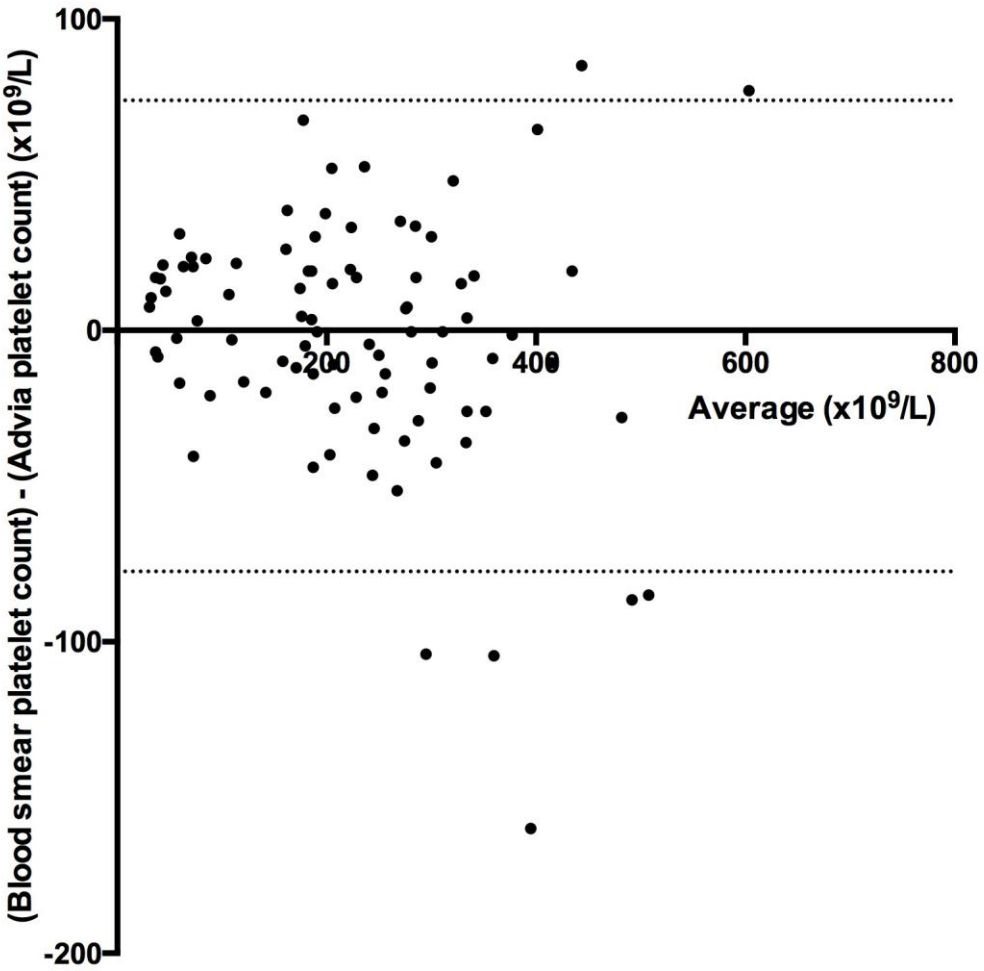

Figure 4.12: Bland Altman plot of two counting methods (blood smear estimation and Advia 120) in the CKCS (n=89).
The difference of platelet values (blood smear-Advia 120) (y-axis) is plotted against the mean value (x-axis). The middle solid line is the mean of the difference. The dotted lines represent the 95% limits of agreement (-77.43 to 73.81). The bias (SD) was -1.82 (38.58).

The correlation between platelet counts obtained by blood smear estimation and Advia 120 was analysed using Spearman's rank correlation (Figure 4.13). There was a significant association (r = 0.9633 (0.94-0.98), P < 0.0001).

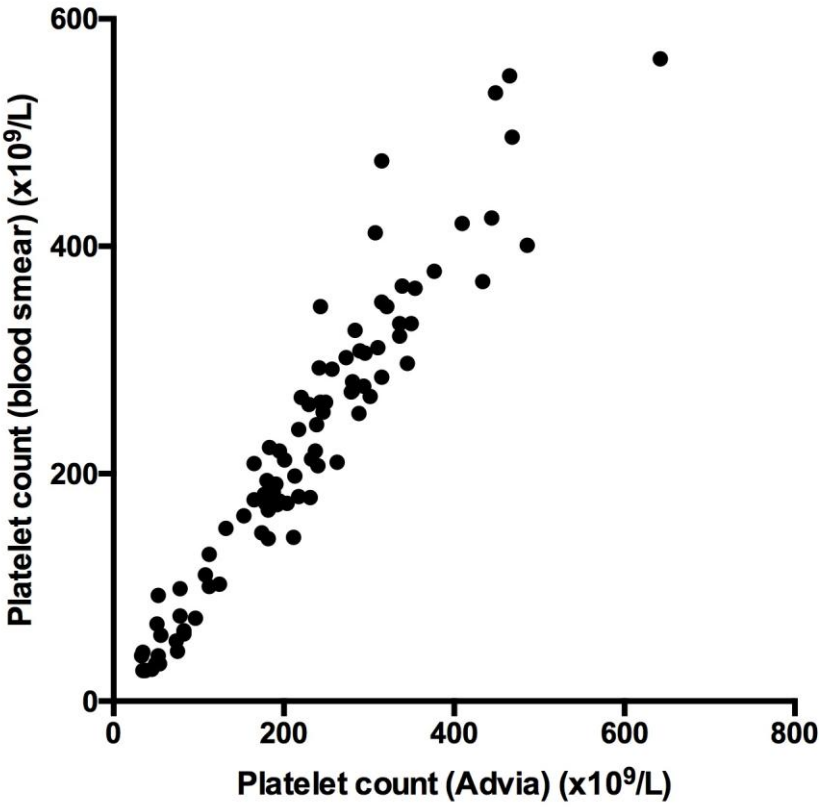

Figure 4.13: Correlation between blood smear estimate and Advia 120 platelet counts

4.4 Selected haematological and platelet function indices

4.4.1 Plateletcrit

Plateletcrit data for the CKCS and control dogs are summarised in Table 4.19 and Figure 4.14. The PCT was significantly lower in the CKCS compared to control dogs (CKCS: median 0.24%, IQR 0.19-0.29%; control dogs: median 0.27%, IQR 0.23-0.36%; $P < 0.011$; Mann Whitney), but a large amount of overlap between the CKCS and control dog results. Despite a large number of CKCS with thrombocytopenia, the majority of CKCS had PCT within canine reference interval (0.13-0.4%) (Kelley et al., 2014).

Plateletcrit (%)	CKCS	Control dogs
Median	0.24	0.27
Range	0.7-0.48	0.11-0.61
IQR	0.19-0.29	0.23-0.36

Table 4.19: Plateletcrit results of the CKCS (n=89) and control dogs (n=39).

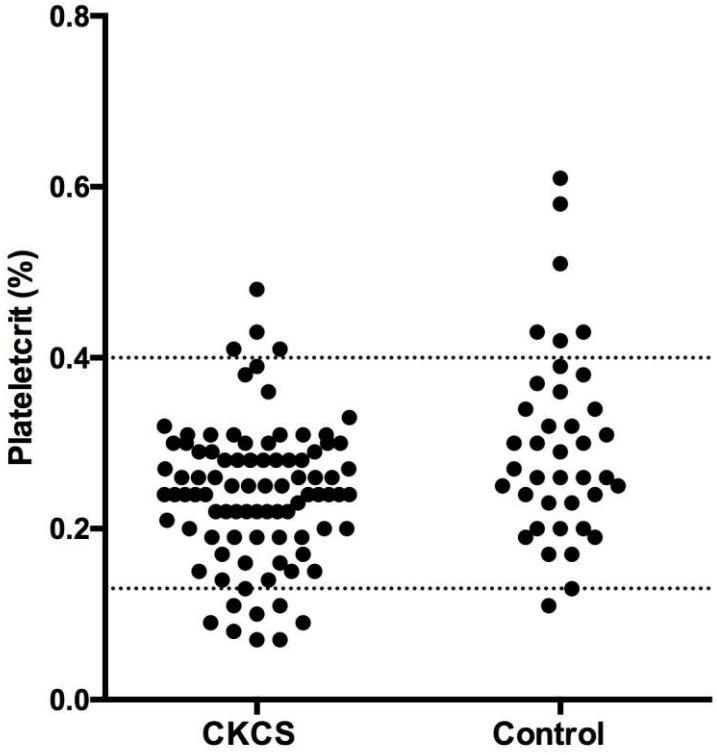

Figure 4.14: Plateletcrit result of each CKCS (n=89) and control dog (n=39).
The dotted lines represent a reference interval of 0.13-0.4% (Kelley et al. 2014).

4.4.2 Mean platelet volume

The MPV data for the CKCS and control dogs are summarised in Table 4.20 and Figure 4.15. The MPV was significantly higher in the CKCS compared to control dogs (CKCS: median 11.2 fL, IQR 8.8-16.1 fL; control dogs: median

8.6 fL, IQR 8.0-9.1 fL; P < 0.001; Mann Whitney). The reported MPV reference interval is 8.56-14.41 fL (Moritz et al. 2004).

MPV (fL)	CKCS	Control dogs
Median	11.2	8.6
Range	7.1-29.4	6.8-11.7
IQR	8.8-16.1	8.0-9.1

Table 4.20: Mean platelet volume results of the CKCS (n=89) and control dogs (n=39).

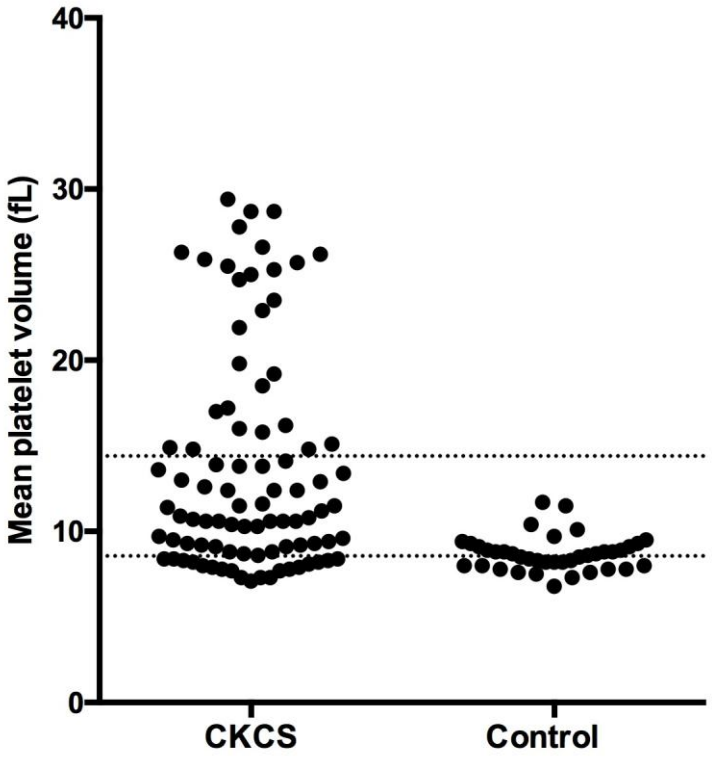

Figure 4.15: Mean platelet volume result of each CKCS (n=89) and control dog (n=39).
The dotted lines represent a reference interval of 8.56-14.41 fL (Moritz et al. 2004).

The percentage of large platelets in both CKCS and control dogs, as determined by MPV, is displayed in Figure 4.16. Based upon a definition of

greater than 30% circulating large platelets, 19 of the 89 CKCS were classified as having macrothrombocytosis when assessed by the Advia 120 (Figure 4.16). Four of these 19 dogs had greater than or equal to 70% circulating large platelets. When macrothrombocytosis was assessed by a different methodology, blood smear assessment, five of 89 CKCS were classified as having macrothrombocytosis. Two of these 5 dogs were classified as having 70% or more macrothrombocytes by the Advia 120.

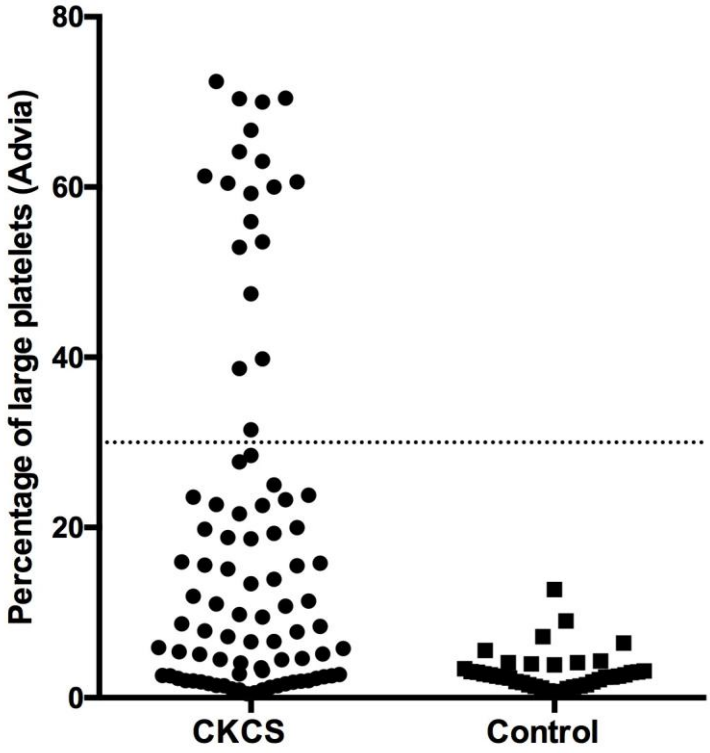

Figure 4.16: Percentage of large platelets (as assessed by the Advia 120) for each of the CKCS (n=89) and control dogs (n=39). Each data point above the dotted line represents a CKCS with greater than 30% circulating large platelets (n = 19).

The MPV was inversely related to platelet count in the CKCS (Figure 4.17) but not in the control dogs; the latter group had similar MPV across platelet counts (Figure 4.18).

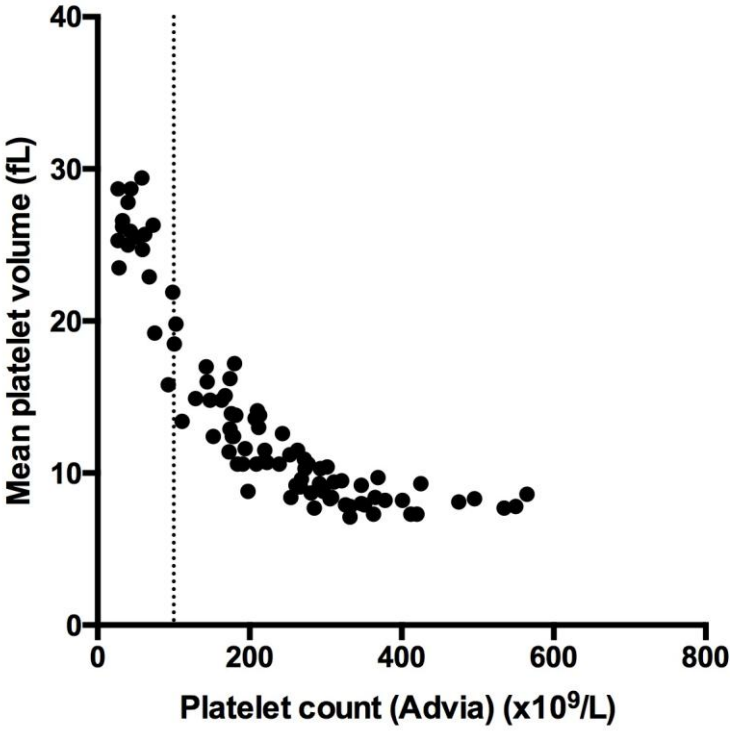

Figure 4.17: Graph displaying the relationship between MPV and platelet count for 89 CKCS.

The dotted line indicates a platelet count of 100 X 10^9/L.

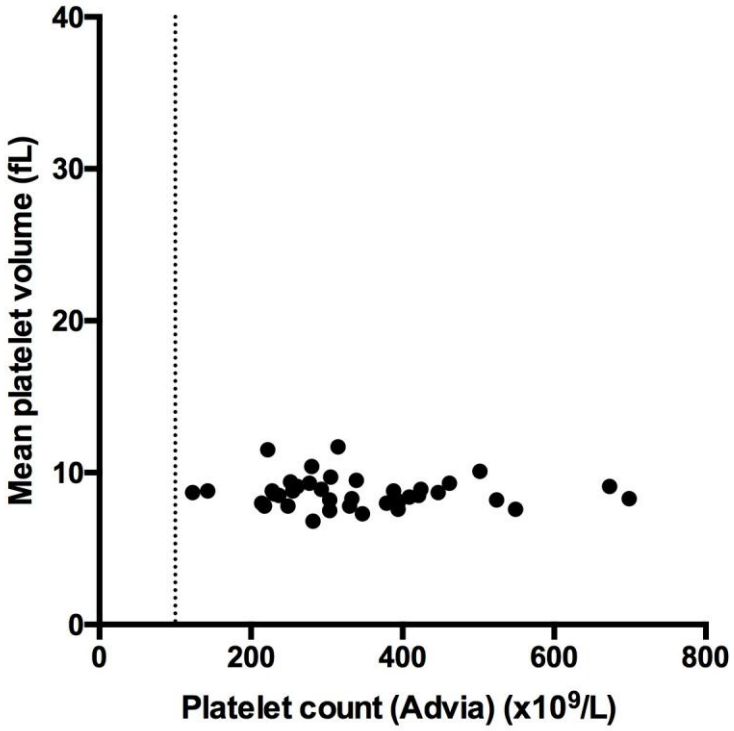

Figure 4.18: Graph displaying the relationship between MPV and platelet count for 39 control dogs.
The dotted line indicates a platelet count of 100 X 10^9/L.

Two control dogs had a platelet count less than 200 x 10^9/L (123 x 10^9/L and 143 x 10^9/L) but MPV within reference interval (8.56 and 14.4 fL respectively). Both dogs were Greyhounds, a breed also recognised for inherited thrombocytopenia, but not macrothrombocytosis.

4.4.3 Platelet volume distribution width

The PDW data for the CKCS and control dogs are summarised in Table 4.21 and Figure 4.19. The PDW was significantly higher in the CKCS compared to control dogs (CKCS: median 59.7%, IQR 52.4-68.5%; control dogs: median 57.4%, IQR 53.2-59.3%; P = 0.044; Mann Whitney), with a large amount of overlap between groups. The reported PDW reference interval is 55.71-66.90% (Moritz et al. 2004).

Platelet volume distribution width (%)	CKCS	Control dogs
Median	59.7	57.4
Range	44.3-78.2	44-67.2
IQR	52.4-68.5	53.2-59.3

Table 4.21: Platelet volume distribution width results of the CKCS (n=89) and control dogs (n=39).

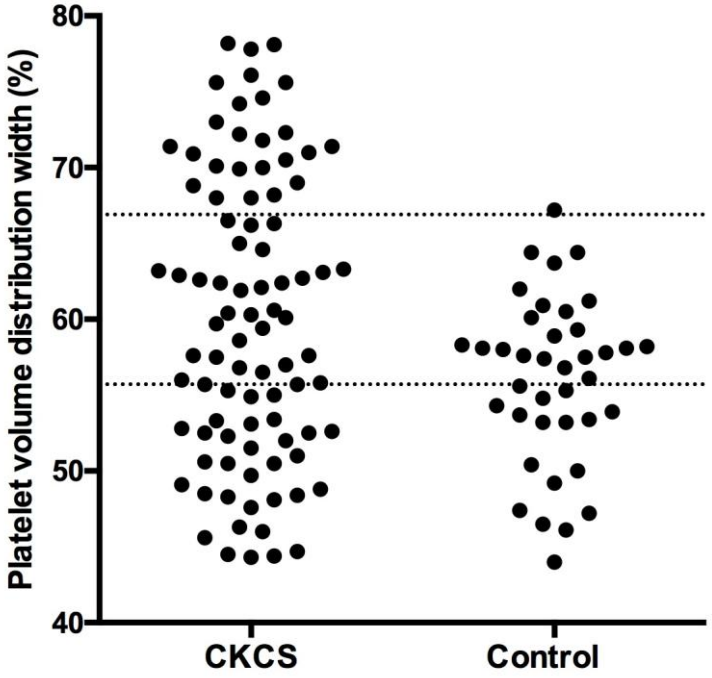

Figure 4.19: Platelet volume distribution width of each CKCS (n=89) and control dog (n=39).
The dotted lines represent a reference interval of 55.71-66.9% (Moritz et al. 2004).

4.4.4 Advia 120 large platelet count

The Advia 120 large platelet data for the CKCS and control dogs are summarised in Table 4.22.

The large platelet count was significantly higher in the CKCS compared to control dogs (CKCS: median 20 x 10^9/L, IQR 9-30 x 10^9/L; control dogs: median 8 x 10^9/L, IQR 4.5-13 x 10^9/L; P < 0.001; Mann Whitney).

Advia 120 large platelet count (x 10^9/L)	**CKCS**	**Control dogs**
Median	20	8
Range	2-47	2-40
IQR	9-30	4.5-13
Advia 120 large platelet percent (%)	**CKCS**	**Control dogs**
Median	9.78	2.55
Range	0.48-72.41	0.71-12.7
IQR	2.6-24.1	1.53-3.87

Table 4.22: Advia 120 large platelet results of the CKCS (n=89) and control dogs (n=39).

4.4.5 Assessment of platelet clumping – Cavalier King Charles spaniel

Platelet aggregates were visually identified in 44 of 89 samples. Number of platelet aggregates was judged as sparse, moderately frequent, and numerous in 26, 9, and 9 blood films, respectively. Aggregate size was classified as small, medium, or large in 14, 16, and 14 samples, respectively. In the 18 CKCS with thrombocytopenia, sparse, moderately frequent, and numerous numbers of aggregates were identified in 3, 3, and 5 samples, respectively. Small, medium, and large aggregates were identified in 7, 2, and 2 samples, respectively. No platelet aggregates were identified in the remaining 7 CKCS. There was no significant difference in closure time, MPC concentration, or PCDW between CKCS with and without platelet aggregates (P = 0.095; Student t test) or between categories for number of aggregates (P = 0.134; ANOVA) or size of platelet aggregates (P = 0.06; ANOVA).

Platelet clumping results in the CKCS using two methods (visual clump assessment and the Advia 120 clump flag) are summarised in Table 4.23.

Visual clump size	**Visual clump number**	**Visual assessment results** (number of dogs)	**Advia 120 clump flag +ve (APCC > 300)** (number of dogs)	**Advia 120 clump flag –ve (APCC ≤ 300)** (number of dogs)
3	**3+**	4	2	2
3	**2+**	3	1	2
3	**1+**	7	3	4
2	**3+**	1	1	0
2	**2+**	4	2	2
2	**1+**	11	6	5
1	**3+**	4	4	0
1	**2+**	2	2	0
1	**1+**	8	5	3
N/A	**0**	45	26	19
Total		89	52	37

Table 4.23: Platelet clumping results in the CKCS using visual clump assessment and the Advia 120 clump flag (n=89).

To determine how the automated platelet clump flag compared with visual detection of platelet clumping, the number of samples with APCC less than or equal to 300 (Advia 120 clump flag –ve) or greater than 300 (Advia120 clump flag +ve) and those with or without visual detection of platelet clumps were compared using Kappa statistics. For this comparison visual platelet scores were dichotomised into “visual clumping” (size and number 1 through to 3+) and “no clumping” (number 0) (Table 4.24).

	Advia 120 clump flag +ve	**Advia 120 clump flag -ve**	Total
Visual clumping	26	18	44
No visual clumping	26	19	45
Total	52	37	89

Table 4.24: Visual platelet scores (clumping/no clumping) against the Advia 120 platelet clump flag in the CKCS (n=89).

There were 45/89 observed agreements. The Kappa statistic (95% confidence interval) was 0.013 (-0.191 to 0.217), consistent with a poor strength of agreement. The standard error of Kappa was 0.104. The Advia 120 flagged clumps in 26 of 45 samples without visually detected clumps (specificity of 42%) and failed to flag clumps in 18 of 44 samples in which platelet clumps were detected visually (sensitivity of 59%).

Subsequently, clump size and clump number (Table 4.25 and 4.26 respectively) were individually compared with the results of the Advia 120 clump flag. The results demonstrate that an increase in clump size does not result in an improved ability of the APCC to detect clumps.

Clump size	**Advia 120 clump flag +ve**	**Advia 120 clump flag -ve**
1	11	3
2	9	7
3	6	8

Table 4.25: Clump size (based on visual assessment) compared with the Advia 120 platelet clump flag status in the CKCS.

Clump number	Advia 120 clump flag +ve	Advia 120 clump flag -ve
1+	14	12
2+	5	4
3+	7	2

Table 4.26: Clump number (based on visual assessment) compared with the Advia 120 platelet clump flag status in the CKCS.

4.4.6 Closure time – Cavalier King Charles spaniel

Closure time was recorded for 76 of 89 CKCS. Closure time was unavailable for 12 CKCS because of failure to perform duplicate assays with the PFA-100 and for 1 CKCS because it had an HCT less than 0.35 L/L. The median closure time was 73 seconds (IQR, 62 to 102 seconds; Table 4.27). The reported reference interval for CT is 52-86 seconds (Callan and Giger 2001). Closure time was greater than the upper limit of the reference interval for 27 CKCS.

The closure time data for the CKCS is summarised in Table 4.27 and Figure 4.20.

Closure time (seconds)	CKCS
Median	73
Range	52-210.5
IQR	62-102

Table 4.27: Closure times in the CKCS (n=76).

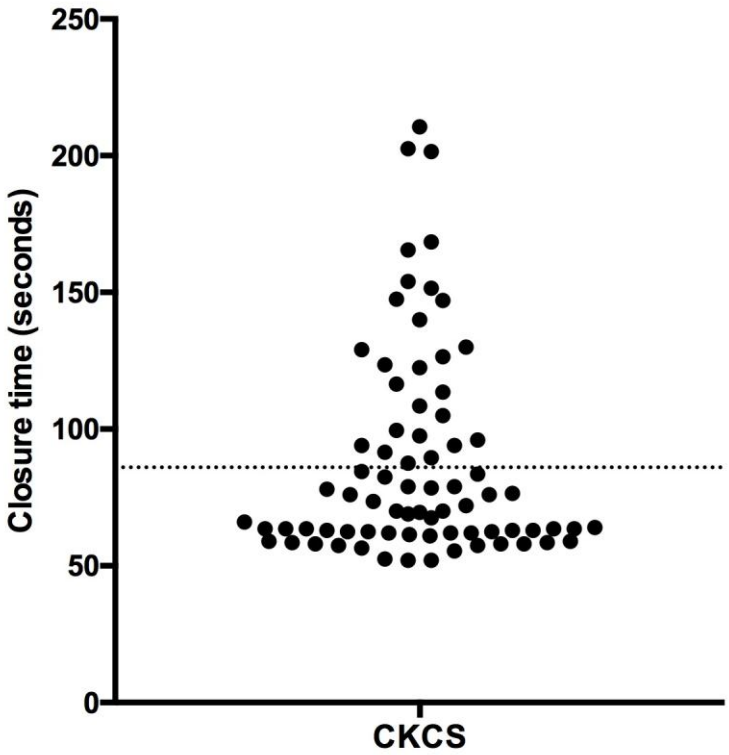

Figure 4.20: Closure time result in the CKCS (n=76).
The dotted line represents a previously reported upper end of the reference interval limit of 86 seconds (Callan et al. 2001). Twenty seven (35.5%) closure time results were above the reference interval.

4.4.7 Mean platelet component concentration

The MPC concentration data for CKCS and control dogs are summarised in Table 4.28 and Figure 4.21. The MPC concentrations were significantly higher in the CKCS compared to control dogs (CKCS: mean 209 g/L, 95% CI of the mean 207-211 g/L; control dogs: mean 204 g/L, 95% CI of the mean 199-208 g/L; P = 0.017; Student *t* test). There was a large amount of overlap between groups.
The reported MPC concentration reference interval is 140-186.3 g/L (Moritz et al. 2004).

MPC (g/L)	CKCS	Control dogs
Mean	209	204
Range	184-241	168-233
95% CI of the mean	207-211	199-208

Table 4.28: Mean platelet component concentration results of the CKCS (n=89) and control dogs (n=39).

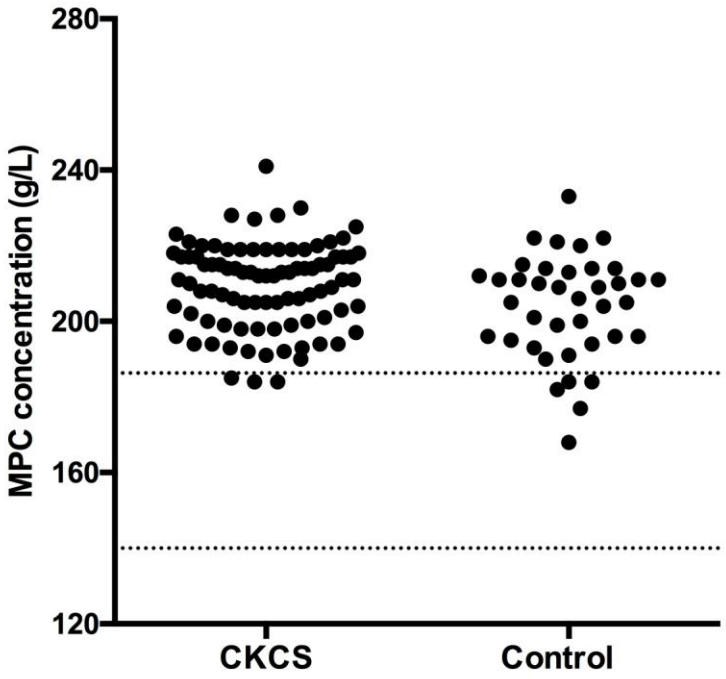

Figure 4.21: Mean platelet component concentration of each CKCS (n=89) and control dog (n=39).
The dotted lines represent a reference interval of 140-186.3 g/L (Moritz et al. 2004).

4.4.8 Platelet component distribution width

The PCDW data for the CKCS and control dogs are summarised in Table 4.29 and Figure 4.22. The PCDW was significantly lower in the CKCS compared to control dogs (CKCS: mean 41 g/L, 95% CI of the mean 39-43 g/L; control dogs: mean 54 g/L, 95% CI of the mean 50-57 g/L; $P < 0.001$;

Student *t* test), with a large amount of overlap between groups. The reported PCDW reference interval is 45.8-70.5 g/L (Moritz et al. 2004).

PCDW (g/L)	CKCS	Control dogs
Mean	41	54
Range	27.4-68.8	35.2-75.7
95% CI of the mean	39-43	50-57

Table 4.29: Platelet component distribution width results of the CKCS (n=89) and control dogs (n=39).

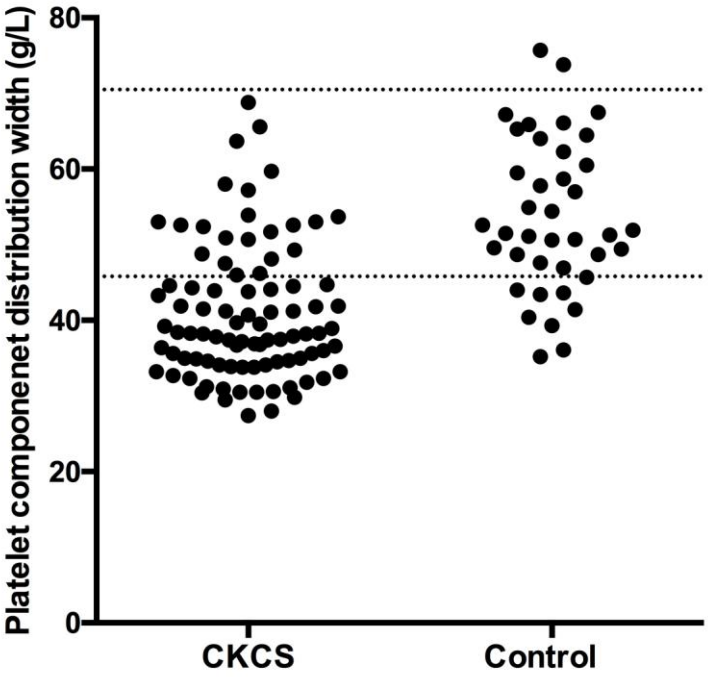

Figure 4.22: Platelet component distribution width of each CKCS (n=89) and control dog (n=39).
The dotted lines represent a reference interval of 45.8-70.5 g/L (Moritz et al. 2004).

4.4.9 Haematocrit

The HCT data for the CKCS and control dogs are summarised in Table 4.30 and Figure 4.23. The HCT values were significantly lower in the CKCS compared to control dogs (CKCS: mean 0.41 L/L, 95% CI of the mean 0.4-0.42 L/L; control dogs: mean 0.5 L/L, 95% CI of the mean 0.48-0.52 L/L; P <

0.001; Student *t* test). The HCT reference interval is 0.42-0.62 L/L (Moritz et al., 2004).

Haematocrit (L/L)	CKCS	Control dogs
Mean	0.41	0.5
Range	0.34-0.47	0.37-0.6
95% CI of the mean	0.40-0.42	0.48-0.52

Table 4.30: Haematocrit results of the CKCS (n=89) and control dogs (n=39).

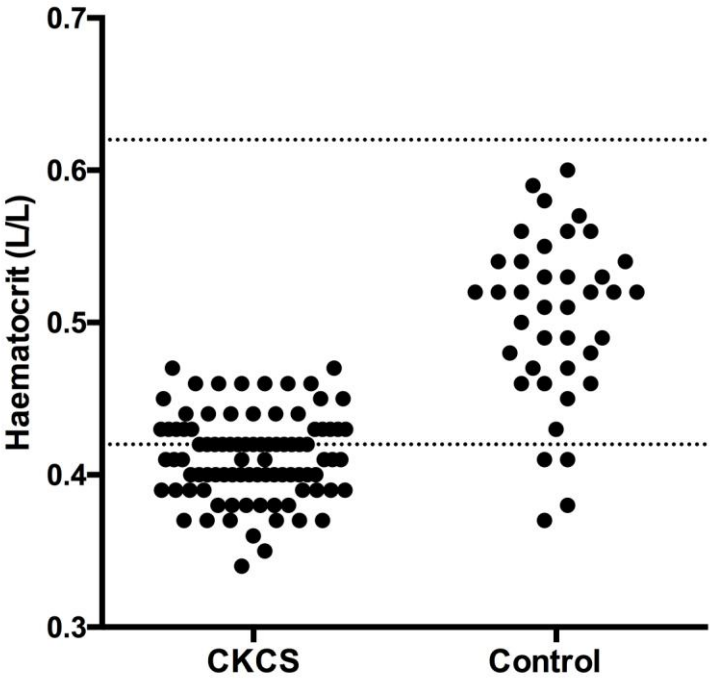

Figure 4.23: Haematocrit of each CKCS (n=89) and control dog (n=39).

The dotted lines represent a reference interval of 0.42-0.62 L/L (Moritz et al. 2004). The majority of the CKCS and control dogs had HCT within reference intervals.

4.4.10 Mean platelet mass

The MPM data for the CKCS and control dogs are summarised in Table 4.31 and Figure 4.24. The MPM was significantly higher in the CKCS compared to control dogs (CKCS: median 2.10 pg, IQR 1.66-2.62 pg; control dogs: median 1.59 pg, IQR 1.51-1.67 pg; $P < 0.001$; Mann Whitney), with a large amount of overlap between groups. The reported MPM reference interval is 1.32-1.92 pg (Moritz et al. 2004).

Mean platelet mass (pg)	CKCS	Control dogs
Median	2.10	1.59
Range	1.36-3.59	1.32-2.23
IQR	1.66-2.62	1.51-1.67

Table 4.31: Mean platelet mass results of the CKCS (n=89) and control dogs (n=39).

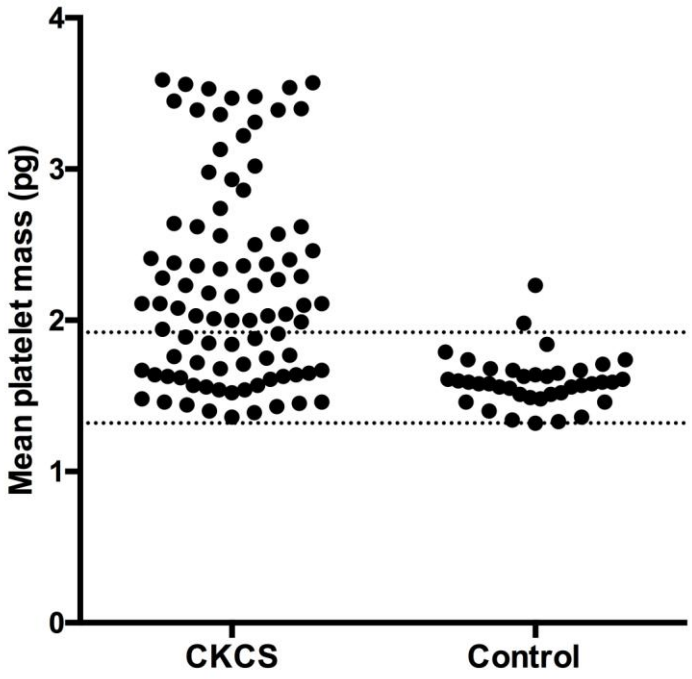

Figure 4.24: Mean platelet mass of each CKCS (n=89) and control dog (n=39).
The dotted lines represent a reference interval of 1.32-1.92 pg (Moritz et al. 2004).

4.4.11 Platelet mass distribution width

The PMDW data for the CKCS and control dogs are summarised in Table 4.32 and Figure 4.25. The PMDW was significantly higher in the CKCS compared to control dogs (CKCS: median 0.87 pg, IQR 0.67-1.0 pg; control dogs: median 0.61 pg, IQR 0.56-0.65 pg; $P < 0.001$; Mann Whitney), with a large amount of overlap between groups. The reported PMDW reference interval is 0.51-0.84 pg (Moritz et al. 2004).

Platelet mass distribution width (pg)	**CKCS**	**Control dogs**
Median	0.87	0.61
Range	0.49-1.31	0.46-0.86
IQR	0.67-1.00	0.56-0.65

Table 4.32: Platelet mass distribution width results of the CKCS (n=89) and control dogs (n=39).

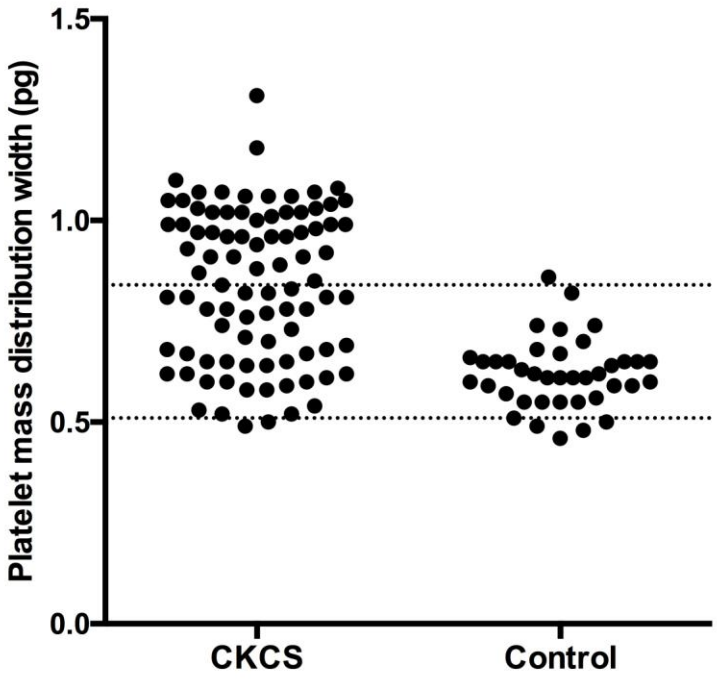

Figure 4.25: Platelet mass distribution width of each CKCS (n=89) and control dog (n=39).
The dotted lines represent a reference interval of 0.51-0.84 pg (Moritz et al. 2004).

4.5 Association analysis

Platelet activation and function has the potential to be affected by turbulent high-velocity blood flow and fluid shear stress associated with valvular heart disease. Association analysis was performed to investigate platelet activation and function in dogs with varying severity of subclinical mitral regurgitation.

4.5.1 The association of closure time with heart disease and selected haematological indices

The association of closure time with measures of CVHD severity, platelet count (Advia 120), HCT, age, sex and bodyweight was explored using multiple regression analysis.

Complete data were available for 76 CKCS (Table 4.33). A model with 5 variables best explained the variation in closure time, with regurgitant jet size exerting the largest effect. The LA:Ao, age, sex, body weight, and HCT had no significant effect on closure time in the multiple linear regression analyses. Closure times differed significantly ($P < 0.001$; ANOVA) among regurgitant jet sizes; CKCS with a jet size greater than 50% had a significantly ($P < 0.001$; least squares mean) longer closure time than did CKCS with a jet size less than 15% or CKCS with a jet size of 15% to 50% (Table 4.34; Figure 4.26).

Variable	**Explanatory variable**	**Partial R^2**	**Model R^2**	**C_p statistic**	**P value**
Closure time (n=76)	Regurgitant jet size	0.61	0.61	35.2	< 0.001
	LVDDN	0.06	0.67	23.2	0.001
	Murmur grade	0.03	0.70	16.7	0.009
	Platelet count	0.02	0.72	13.0	0.026
	LVDSN	0.02	0.74	8.6	0.015
	Model	n/a	0.74	8.6	0.015
MPC concentration (n=89)	Platelet count	0.24	0.24	-1.8	< 0.001
	Model	n/a	0.24	-1.8	< 0.001
PCDW (n=89)	Platelet count	0.21	0.21	1.25	< 0.001
	Sex	0.04	0.25	4.86	0.030
	Model	n/a	0.25	4.86	0.030

Table 4.33: Results of multiple regression analyses of data for CKCS (n=76).

Severity group	Regurgitant jet size (%)*	n	Closure time (s)
Absent-minimal	< 15	43	67 (64-70)
Mild	15 to 50	12	78 (65-92)
Moderate-severe	> 50	21	138 (122-155)†

Table 4.34: Regurgitant jet size and corresponding closure time values (mean [95% CI]) for 76 CKCS.

*Regurgitant jet size was < 15%, 15% to 50% and > 50% for 52, 14, and 23 CKCS respectively; in the 89 CKCS, however, CT was not determined in 13 dogs, hence the results for 76 CKCS in this table. † Value differs significantly ($P < 0.001$) from the value for the other regurgitant jet sizes.

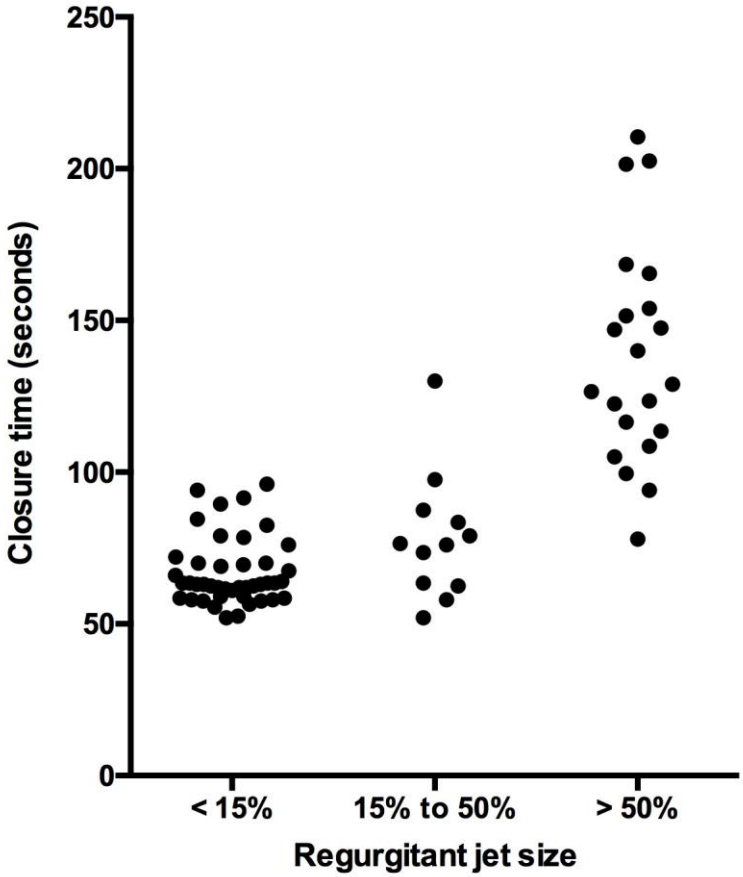

Figure 4.26: Graph displaying closure time for 76 CKCS that had regurgitant jet size < 15% (n = 43), 15% to 50% (n = 12), and > 50% (n = 21).

The closure time was significantly (*P* < 0.001; least squares mean) longer for CKCS with regurgitant jet size > 50%, compared with closure time for dogs with regurgitant jet size < 15% or 15% to 50%.

4.5.2 The association of mean platelet component concentration and platelet component distribution width with heart disease and selected haematological indices

The association of MPC concentration and PCDW with measures of CVHD severity, platelet count (Advia 120), HCT, age, sex and bodyweight was explored using multiple regression analysis.

Complete data were available for 89 CKCS. The model that best explained the variation in MPC concentration included only platelet count (Table 4.33). The model that best explained the variation in PCDW included platelet count and sex. The relationship between MPC concentration and platelet count (Figure 4.27) and between PCDW and platelet count (Figure 4.28) are graphed.

Five of the 7 data points with the highest platelet counts and lowest MPC concentrations represented samples without platelet clumping (Figure 4.27).

There was a significant negative association of MPC concentration with platelet count (R^2 = 0.24, P <0.001) (Table 4.33).

There was a significant positive association of PCDW with the platelet count (R^2 = 0.21, P < 0.001). In addition, sex was found to be a weak component of the model (R^2 = 0.04, P = 0.030) (Table 4.33).

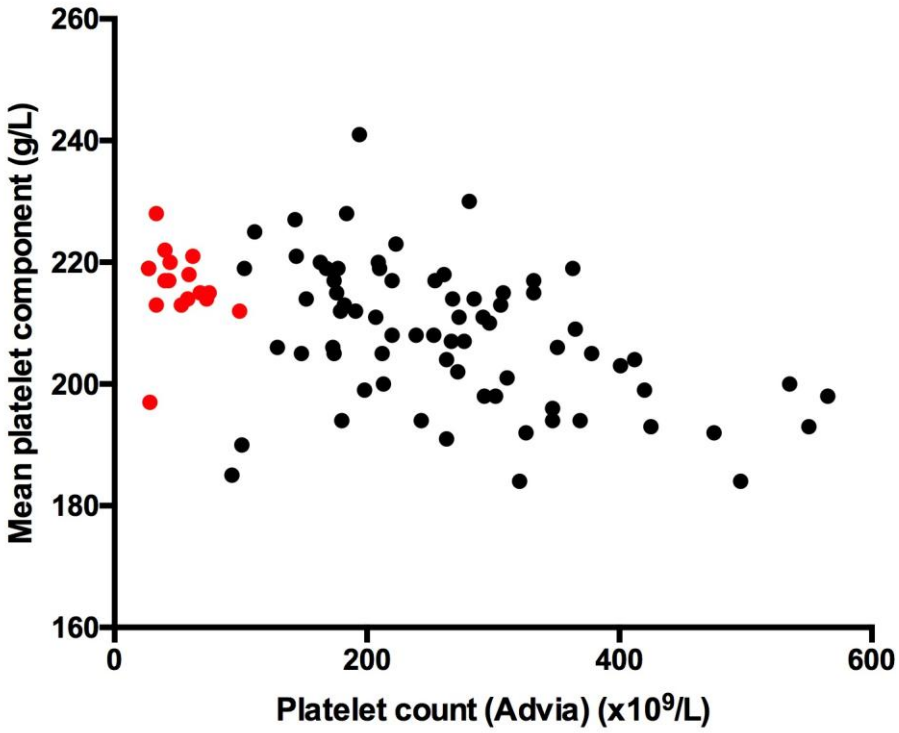

Figure 4.27: Graph displaying the relationship between MPC concentration and platelet count for 89 CKCS.

The red data points represent dogs with a MPV of > 20 fL i.e. the dogs with larger platelets. Multiple regression analysis revealed that a model containing only 1 variable (platelet count) best explained the variation in MPC concentration (model R^2, 0.24; $P < 0.001$).

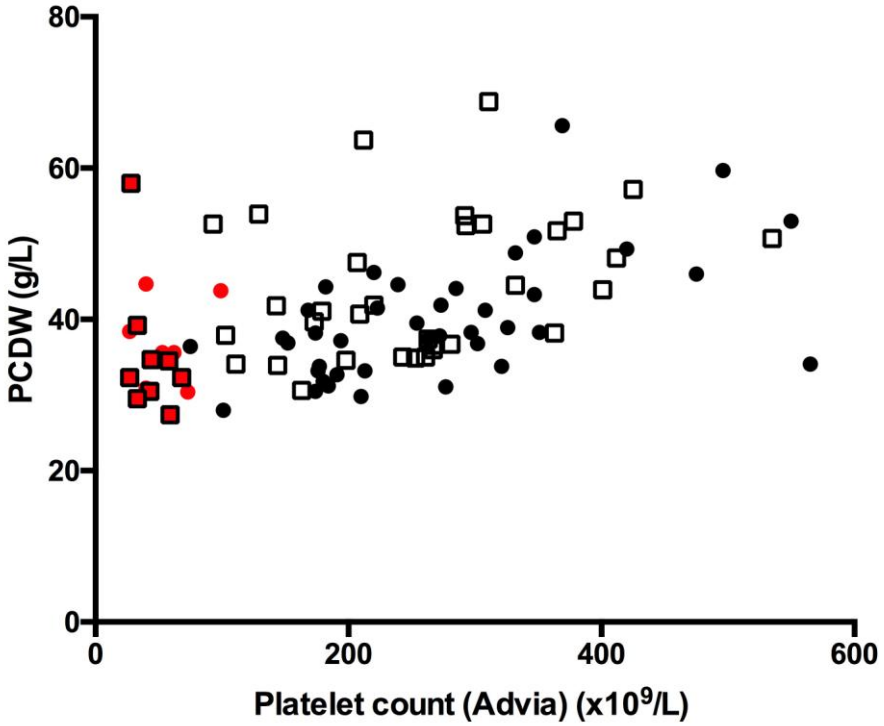

Figure 4.28: Graph displaying the relationship between PCDW and platelet count for 89 CKCS (42 males [white squares] and 47 females [black circles]).

The PCDW increases with increasing platelet count (consistent with increased variation in platelet density with increasing platelet count). The red data points represent dogs with a MPV of > 20 fL i.e. the dogs with larger platelets. Multiple regression analysis revealed that a model containing 2 variables (platelet count and sex) best explained the variation in PCDW (platelet count partial R^2, 0.21; model R^2, 0.25 [P = 0.03]).

4.5.3 The association of mean platelet volume with heart disease and selected haematological indices

The association of MPV with measures of CVHD severity, platelet count (Advia 120), HCT, age, sex and bodyweight was explored using multiple regression analysis.

There was a significant negative association of MPV with the platelet count (R^2 = 0.61, P < 0.001) (Figure 4.17 and Table 4.35).

Age, sex, body weight, HCT and heart disease indices had no significant influence on MPV in the multiple linear regression analyses.

	Partial R-square	Model R-square	P value
Platelet count	0.61	0.61	< 0.001

Table 4.35: Results of MPV multiple regression analysis.

4.5.4 The association of platelet volume distribution width with heart disease and selected haematological indices

The association of PDW with measures of CVHD severity, platelet count (Advia 120), HCT, age, sex and bodyweight was explored using multiple regression analysis.

There was a significant, complex association of PDW with the platelet count (R^2 = 0.05, P = 0.044) (Table 4.36). Figure 4.29 displays the PDW of each dog against platelet count.

Age, sex, body weight, HCT and heart disease indices had no significant influence on PDW in the multiple linear regression analyses.

	Partial R-square	Model R-square	P value
Platelet count	0.05	0.05	0.044

Table 4.36: Results of PDW multiple regression analysis.

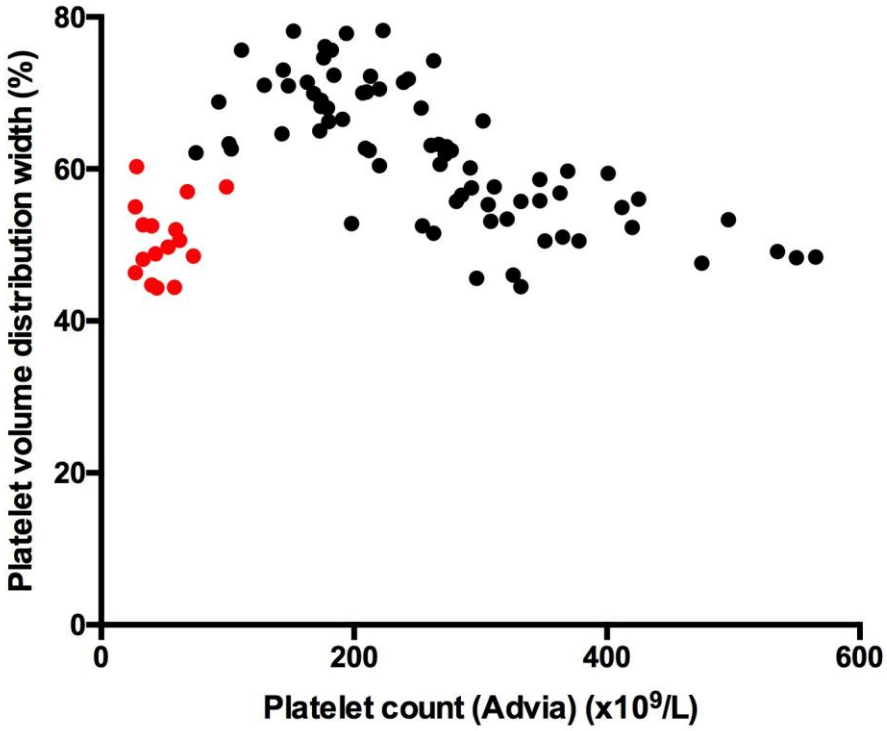

Figure 4.29: Graph displaying the relationship between PDW and platelet count for 89 CKCS.
The red data points represent dogs with a MPV of > 20 fL i.e. the dogs with larger platelets.

4.5.5 The association of mean platelet mass with heart disease and selected haematological indices

The association of MPM with measures of CVHD severity, platelet count (Advia 120), HCT, age, sex and bodyweight was explored using multiple regression analysis.

There was a negative association of MPM with the platelet count (R^2 = 0.80, P < 0.001). In addition jet size was found to be a weak component of the model (R^2 =0.01, P = 0.02) (Table 4.37). Figure 4.30 displays the MPM of each dog against platelet count.
Age, sex, body weight, HCT and heart disease indices (excluding jet size) had no significant influence on MPM in the multiple linear regression analyses.

	Partial R-square	Model R-square	P value
Platelet count	0.80	0.80	< 0.001
Jet size	0.01	0.81	0.022

Table 4.37: Results of MPM multiple regression analysis.

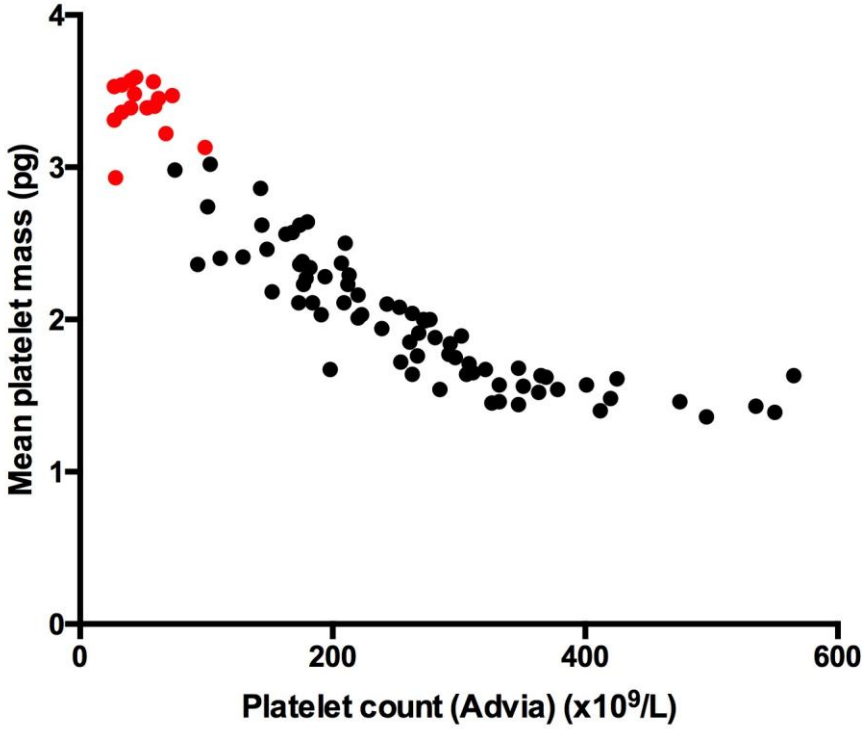

Figure 4.30: Graph displaying the relationship between MPM and platelet count for 89 CKCS.

MPM is higher at lower platelet counts, and that MPM decreases as platelet count increases. The red data points represent dogs with a MPV of > 20 fL i.e. the dogs with larger platelets.

To test the hypothesis that larger platelets have more granules than smaller platelets (since lower platelet counts are associated with larger platelet size), MPM was subsequently plotted against MPV (Figure 4.31).

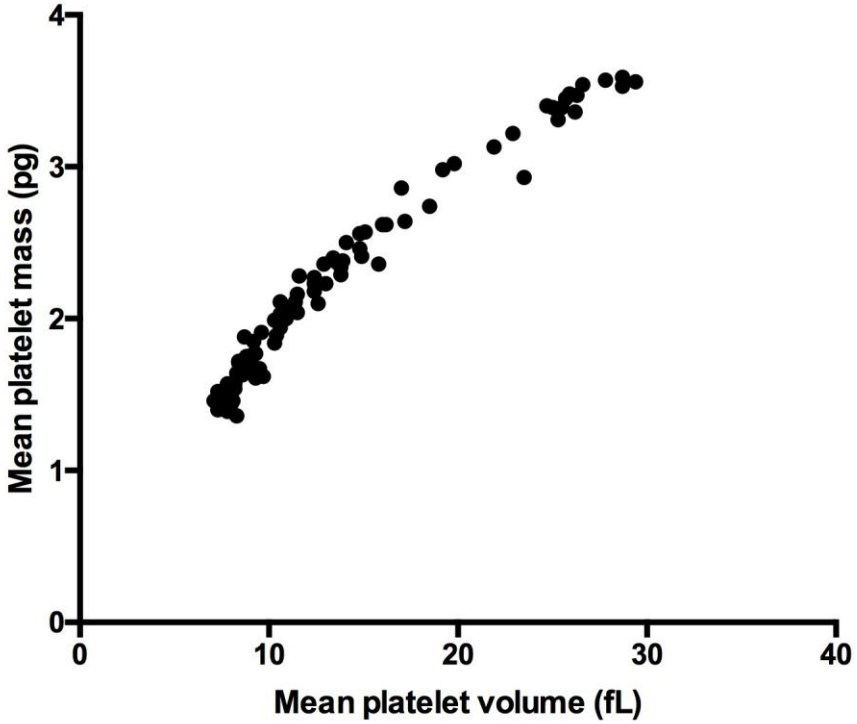

Figure 4.31: Graph displaying the relationship between MPM and MPV for 89 CKCS.

Visual inspection of the graph revealed a positive relationship between MPM and MPV.

4.5.6 The association of platelet mass distribution width with heart disease and selected haematological indices

The association of PMDW with measures of CVHD severity, platelet count (Advia 120), HCT, age, sex and bodyweight was explored using multiple regression analysis.

There was a significant complex association of PMDW with the platelet count (R^2 = 0.53, P < 0.001). In addition murmur grade was found to be a weak component of the model (R^2 = 0.03, P = 0.013) (Table 4.38). Figure 4.32 displays the PMDW of each dog against platelet count.

Age, sex, body weight, HCT and heart disease indices (excluding murmur grade) had no significant influence on PMDW in the multiple linear regression analyses.

	Partial R-square	Model R-square	P value
Platelet count	0.53	0.53	< 0.001
Murmur grade	0.03	0.57	0.013

Table 4.38: Results of PMDW multiple regression analysis.

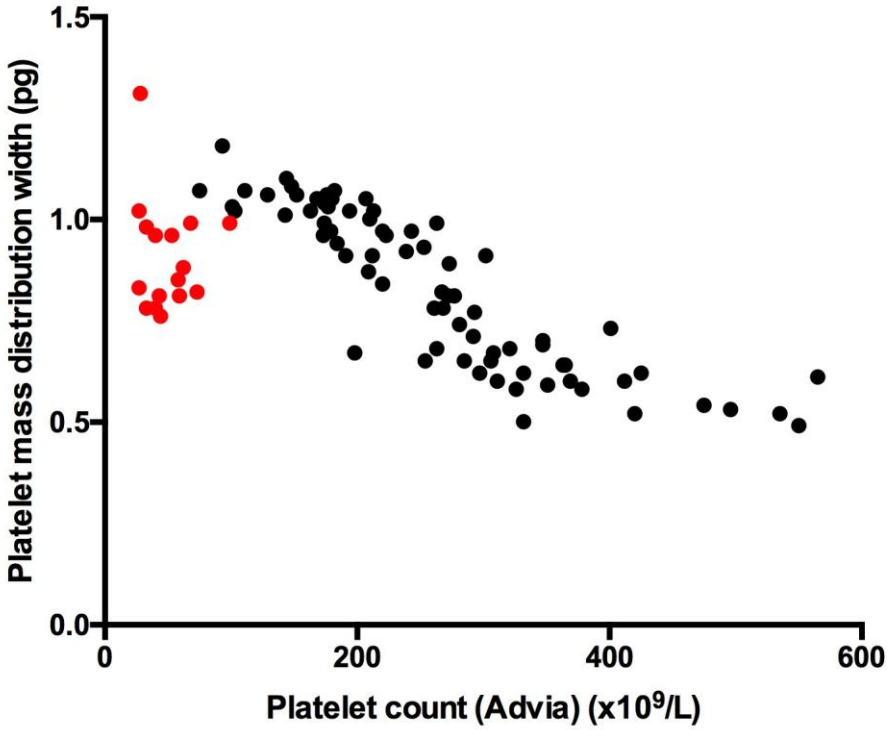

Figure 4.32: Graph displaying the relationship between PMDW and platelet count for 89 CKCS.

The red data points represent dogs with a MPV of > 20 fL i.e. the dogs with larger platelets.

5 Discussion

The primary objective of this present study was to investigate associations between markers of heart disease severity and platelet function or activation. Although 5 variables significantly affected closure time, the regurgitant jet size exerted by far the largest effect, as previously described (Tarnow et al. 2003). This may have reflected quantitative and qualitative changes in VWF rather than an alteration of intrinsic platelet function (Tarnow et al. 2004).

Comparison across jet size categories showed that the closure time of CKCS with jet size greater than 50% was significantly higher than that of CKCS with jet size less than 15% and 15-50% ($P < 0.0001$). These results are similar to a previous study (Tarnow et al. 2003), however in the latter study the closure time of CKCS with jet size greater than 50% was significantly higher than that of CKCS with jet size less than 15% but not dogs with jet size 15-50%. In addition, in the aforementioned study the closure time of CKCS with jet size 15-50% was different to CKCS with closure time less than 15%.

There was no association between closure time and age, sex, body weight, LA:Ao and HCT. The lack of an association between closure time and age, sex, body weight and LA:Ao is in line with the results of a previous study (Tarnow et al. 2003). The lack of an association between closure time and HCT, however, contradicts the results of Tarnow et al. (2003) and is difficult to explain. This result is not likely due to exclusion of dogs with HCT less than 0.35L/L in this present study because only one dog was excluded for this reason.

By contrast, none of the indicators of heart disease severity significantly affected MPC concentration or PCDW. This suggested that platelet activation was not a feature of valvular heart disease in CKCS, which supports results of thromboelastography and evaluation of P-selectin expression and thromboxane concentrations (Tarnow et al. 2005). In humans with cardiac disease, valvular changes or associated alterations in blood flow are

believed to initiate platelet adherence and aggregation, which potentially leads to thromboembolic complications (Barnett et al. 1976, Kostuk et al. 1977). Significantly increased platelet aggregation responses and plasma concentrations of PF4 and BTG have been described for humans with combined MVP and MVR, compared with results for healthy control subjects (Walsh et al. 1981, Tse et al. 1997). Similarly, an increase in MPV, which is suggestive of platelet activation, has been observed in a variety of human cardiac diseases, including MVP (Varol et al. 2009, Icli et al. 2013). Reasons for the apparent differences in platelet activation between heart disease in dogs and humans remain unclear. Presumably, platelet reactivities or pathogenic mechanisms that incite activation differ between the species. Mean platelet component concentration was the platelet activation marker of choice in the present study because it was theorised that decreased platelet density (i.e decreased MPC concentration) may be detectable after the loss of cell-surface P-selectin (Macey et al. 1999) and the results of a previous study (Moritz et al. 2005) suggested that MPC concentration may be a more sensitive indicator of platelet activation than P-selectin. Furthermore it was expected that MPC concentration, being a measure of platelet density would be less affected by the CKCS inherent variation in platelet size, unlike other proposed platelet activation markers, MPV, PDW, MPM and PMDW that increase with platelet size.

Despite the limitations of MPV, PDW, MPM and PMDW as platelet activation markers in the CKCS, the association of these indices with CVHD variables were similarly explored using multiple regression analyses (see Sections 4.5.3 through to 4.5.6). Heart disease indices did not have a significant influence on MPV or PDW in the multiple regression analyses. There was however a weak negative influence of jet size in the MPM multiple regression analysis (possibly consistent with increased platelet degranulation with advancing CVHD); and a weak positive influence of murmur grade in the PMDW multiple regression analysis (possibly consistent with increased variation in platelet granular mass/increased platelet activation with advancing heart disease). Since jet size and murmur grade were only weak components of their respective models however it is important not to over–

emphasis the role played by each. As described above, a limitation in the present study is that the presence of inherently large platelets in the CKCS may reduce the sensitivity of MPV, PDW, MPM and PMDW for the detection of platelet activation in the CKCS.

The results of the present study support that of previous studies which reported that CVHD is common in the CKCS. There was at least minimal MVR (on colour-flow Doppler echocardiography) in the vast majority (86 of 89 i.e. 97%) of CKCS, and a left apical systolic murmur in 47% of CKCS aged 6 years or less. These results are similar to previous studies which report ultrasonographic evidence of MVP in 97% of CKCS greater than three years of age (Pedersen et al. 1999a) and a murmur in half of all CKCS by age 6 - 7 years (Pedersen et al. 1999a, Chetboul et al. 2004).

Because of the high proportion of dogs with MVR, lack of a significant association between indicators of heart disease severity and indices of platelet activation could also have been explained by the activation of platelets in almost all dogs. This was considered unlikely because the MPC concentration was higher in CKCS, compared with concentrations in healthy control dogs, which suggested a lack of platelet activation. It was also possible that activated platelets were removed from circulation, leaving a residual population of platelets with a relatively higher MPC concentration. However, platelet activation in humans is proportional to the severity of MVR (Tse et al. 1997, Icli et al. 2013), and in the present study, a relationship between indicators of heart disease severity and MPC concentration would have been expected, regardless of the direction of change.

The anticoagulant EDTA was chosen for analysis with the Advia 120, because it is the preferred anticoagulant for determining differential blood counts with haematology analyses, principally for its cell preservation properties (Macey et al. 2002). In people however, the latter study concluded that an anticoagulant consisting of a combination of citrate, theophylline, adenosine and dipyridamole (CTAD) and EDTA was best for platelet activation assessment. More specifically they concluded that blood collected

into combined CTAD and EDTA, stored at 4 degrees Celcius, and analysed between 60 and 180 minutes later, facilitated maximum platelet sphering without concurrent artefactual platelet activation (Macey et al. 2002). In dogs the use of combined CTAD and EDTA has not been assessed and the optimal anticoagulant for assessment of MPC concentration has not been determined.

The prevalence of thrombocytopenia, in the present study (i.e. 21%, as assessed by blood smear estimate or Advia 120), was similar to that reported in previous studies (i.e. 22-56%, as assessed by manual and blood smear estimate platelet counts) (Eksell et al., 1994, Olsen et al., 2001, Pedersen et al., 2002, Cowan et al., 2004, Olsen et al., 2004, Singh and Lamb, 2005).

The lower prevalence of macrothrombocytosis in CKCS in the present study (7%, as assessed by blood smear) compared to that previously reported (30 and 33%, as assessed by blood smear and electron microscopy respectively) (Cowan et al., 2004, Singh and Lamb, 2005), may reflect a true lower prevalence of macrothrombocytosis in the cohort of CKCS assessed, or subjectivity of the blood smear classification criterion. On the other hand, the higher prevalence of macrothrombocytosis as assessed by the Advia 120 in the present study (22%), is likely due to the Advia 120's less stringent method of macrothrombocyte assessment.

The most stringent method of macrothrombocyte assessment involves only counting platelets as large as or larger than an erythrocyte on a blood smear. Since canine erythrocyte diameter is approximately 7µm (Rizzi et al. 2010), only platelets approximately 7µm or greater in diameter are counted as macrothrombocytes. In contrast, the previous definition of a macrothrombocyte using electron microscopy was less strict, with any platelets with a diameter of 3µm or greater counted as macrothrombocytes (Cowan et al. 2004). The Advia 120 is also less strict than blood smear estimation in classifying macrothrombocytes, counting all platelets with a volume 21 to 60 fL as large platelets (macrothrombocytes); noting that the

lower limits of the Advia 120 reference interval for large platelet volume (21 fL) is approximately one third the lower reference interval for erythrocyte volume (62.7fL) (Moritz et al. 2004). The true prevalence of macrothrombocytopenia could have been achieved by assessment of the mutation (homozygous, heterozygous or clear) in the present population of CKCS.

Two of 39 control dogs had a platelet count of less than 200 x 10^9/L. Both dogs were Greyhounds, a breed known to have a lower reference interval for platelets, similar to other sight hounds. In both dogs, as expected, the MPV was within reference intervals.

Platelet aggregates were visually identified in 44 of 89 (49%) CKCS. In 1 previous study (Koplitz et al. 2001), 26 blood samples were collected by experienced phlebotomists from a jugular vein of cooperative dogs and expediently transferred to anticoagulant. Despite use of this technique, at least mild platelet aggregation was observed in 14 (54%) samples. In theory, platelet aggregation during or after blood collection has the potential to activate platelets and alter MPC concentration. A resultant lower MPC concentration would be expected with a decreasing platelet count, rather than the inverse relationship that was observed in the present study. Alternatively, a lower platelet count and higher MPC concentration could be expected if activated platelets preferentially clumped, which would result in a residual platelet population with a higher MPC concentration. However, MPC concentrations were not significantly different between dogs with and without platelet aggregation (see Section 4.4.5).

This present study showed a poor strength of agreement between the visual detection of platelet clumping and automated platelet clump flag. The APCC flagged platelet clumps in 26/45 samples without visually detected clumps and failed to flag platelet clumps in 18/44 samples with visually detected clumps in smears. An increased clump size did not result in an improved ability of the APCC to detect clumps. Overall, the present study results suggested that the APCC is a poor indicator of clumping in dogs and a blood

smear assessment should be performed to verify the presence or absence of platelet clumping. Only one other canine study (Stokol and Erb 2007) has assessed the validity of APCC for the detection of platelet clumps (using a population of dogs with neoplasia), and reported a poorer sensitivity, but better specificity than that obtained in the present study (see Section 4.4.5). The sensitivity and specificity of the two studies cannot be directly compared because the platelet clumping categories were not standardised.

Several differences were identified between platelet indices in the CKCS and control groups. Unsurprisingly, platelet count was significantly lower, and MPV significantly higher in the CKCS group, which reflects the presence of macrothrombocytopenia in several CKCS. This was supported by higher MPV values in CKCS with lower platelet counts (Figure 4.17).

Macrothrombocytopenia in CKCS is associated with a nonsynonymous single nucleotide polymorphism in the gene encoding β1-tubulin, which is believed to lead to altered proplatelet production by megakaryocytes (Davis et al. 2008). Although the β-1 isolate of β-tubulin is considered to be megakaryocyte specific, it may be upregulated in other tissues under pathological conditions. Although densification of microtubular networks within cardiomyocytes contributes to contractile dysfunction in subjects with experimentally induced heart disease, it is unclear whether this altered β-tubulin structure plays a role in the pathogenesis of naturally occurring CVHD in CKCS (Tsutsui et al. 1993, Koide et al. 2000).

The PCT was significantly lower for the CKCS group. However, the PCT for the optical-based haematology analyser was determined indirectly by use of the equation PCT = platelet count X MPV/10,000. The optical-based haematology analyser used light-scatter signals acquired at 2 angles that were converted into volume and refractive indices via calculation with the Mie light-scatter theory (Briggs et al. 2007). A graphic representation of the 2 light-scatter measurements was created, and platelets were identified in the region corresponding to a volume of 1 to 60 fL and refractive index of 1.35 to 1.40. The platelet-scatter cytogram displayed cells with volumes of 0 to 30 fL.

The large platelet area of the red blood cell-scatter cytogram displayed large platelets with volumes between 31 and 60 fL. The reported platelet count was the sum of platelets and large platelets identified in the platelet- and red blood cell-scatter cytograms, respectively. It has been suggested that the platelet algorithm for the optical-based haematology analyser may deliberately exclude large platelets from analysis if the hypochromic-macrocytic flag results in misclassification of large platelets as small red blood cells, with a resultant lower platelet count and derived PCT (Tvedten et al. 2012). Such misclassification of platelets as a result of macrothrombocytopenia could explain the lower PCT derived for the CKCS. Despite a large number of CKCS with thrombocytopenia, the majority of CKCS had PCT within reference interval, consistent with adequate platelet volume/mass (due to larger platelet size/higher MPV) despite the lower platelet counts. This finding suggests that PCT may aid in the differentiation between inherited macrothrombocytopenia and significant disease-related thrombocytopenia in the CKCS breed.

A greater MPC concentration within CKCS was consistent with interbreed variation. To the author's knowledge, this has not been previously described in dogs. However, lower platelet aggregation responses have been reported in CKCS relative to healthy Beagles, and higher responses have been reported relative to a group of Cairn Terriers, Labrador Retrievers, and Boxers, which suggests that breed factors should be considered when interpreting results of platelet function tests (Cowan et al. 2004, Nielsen et al. 2007). In contrast to the human literature (Giacomini et al., 2001), the present study did not find a decrease in MPC concentration with age.

The MPV of the CKCS in the present study was higher than that of the control dogs, as expected, due to the inherited large platelet size in this breed. The higher PDW in the CKCS compared to the control dogs was consistent with increased variation in platelet size in the CKCS. The higher MPM in the CKCS, compared with the control dogs is likely due to higher granule content per CKCS platelet. The latter result suggests that larger platelets contain more granules than smaller platelets. The PMDW of the

CKCS was significantly higher than that of the control population, consistent with increased variation in granule content in the CKCS population, likely reflecting the presence of both small and large circulating platelets in the CKCS breed. The majority of CKCS and control dogs had HCT values within reference intervals. Interestingly, the CKCS HCT values were tightly clustered around the low to mid limits of the reference interval, whereas the HCT of the control dogs were more evenly distributed across the reference interval. This result most likely represents (low intra-breed) variance in the CKCS group.

Interestingly, platelet count had a small but significant effect on MPC concentration and PCDW in the multiple regression models. The MPC concentration was lower and PCDW higher with increasing platelet counts. Both of these findings suggest greater platelet activation at higher counts. Investigators of a previous study (Olsen et al. 2001), found higher maximal platelet aggregation responses for CKCS with platelet counts greater than 100 x 10^9/L, compared with responses for healthy control dogs and CKCS with less than 100 x 10^9/L. The reasons for these associations are not known. One possible theory is that in the present study, there was a subclinical stimulus, unassociated with heart disease, for concurrent platelet activation and platelet formation in the population of CKCS assessed. It was not due to the fact that there was more clumping at higher platelet counts because 5/7 data points with the highest counts and lowest MPC concentrations represented samples without platelet clumping (see Section 4.5.2). Furthermore this association was not due to larger platelets being denser because there was no association between MPC concentration and MPV. Additional studies are necessary to compare the effects of platelet count and macrothrombocytopenia on markers of platelet activation.

In addition to MPC concentration and PCDW, platelet count had a small but significant influence on other dependent variables including closure time, MPV, PDW, MPM and PMDW in the multiple regression models. For some dependent variables such as PDW and PMDW, the relationship with platelet count was complex and could not be described as simply positive or

negative. The influence of platelet count in the closure time model was weak (partial R^2 = 0.02, P = 0.026), consistent with adequate platelet function despite some very low platelet counts. Similarly in a previous study platelet count had no significant influence on closure time in CKCS (Tarnow et al. 2003), consistent with the breed's larger platelet size and enhanced function per platelet. The negative association of MPV with platelet count was physiologically expected. It reflects the fact that, assuming consistent receptor density, it is the number of TPO receptors, not platelet number that determines platelet production and permits maintenance of PCT within reference intervals (refer to Section 1.3.1.4). The relationship of PDW and platelet count was complex (Figure 4.29). There was least variation in platelet size in CKCS with platelet counts less than 100 x 10^9/L and greater than 250 x 10^9/L, and the most variation in platelet size in dogs with platelet counts 100 to 250 x 10^9/L. This result demonstrates that CKCS with the lowest platelet counts (i.e. less than 100 x 10^9/L) and highest counts (i.e. greater than 250 x 10^9/L) have little variation in platelet size (due to a high percentage of large platelets and normal platelets in circulation respectively). Conversely dogs with platelet counts 100 to 250 x 10^9/L are most likely to have a combination of large and normal sized platelets. MPM was higher at lower platelet counts, and MPM decreased as platelet count increased (Figure 4.30). This result was likely due to larger platelets having more granules than smaller platelets (since lower platelet counts are associated with larger platelet size). To test this hypothesis, MPM was subsequently plotted against MPV (Figure 4.31). A linear, positive relationship between MPM and MPV was obtained and confirmed increased granule mass with increased platelet size. A complex relationship between PMDW and platelet count (Figure 4.32) was established. There was lowest PMDW (least variation in mass of platelet component) in CKCS with platelet counts greater than 250 x 10^9/L and platelet size less than 100 x 10^9/L, likely due to the fact that these two groups of CKCS have the least variation in platelet size i.e. either majority small or large platelets respectively. The most variation in mass of platelet component was found in CKCS with platelet counts 100 to 250 x 10^9/L, the population of CKCS most likely to have a combination of large and normal sized platelets.

Dogs in the present study were apparently healthy; however, subclinical diseases could not be excluded and may have affected platelet activation indices in some cases. For example, increased platelet activation has been described in dogs with malignant neoplasia (McNiel et al. 1997), and internal neoplasia could not be excluded in the dogs of the present study without additional diagnostic imaging. Although such unrecognised diseases could have affected platelet activation indices in some dogs, it was thought unlikely to affect results of the present study because of the expected low prevalence of such diseases in dogs with no clinical signs of illness.

Limitations of the present study included inclusion of a small number of CKCS without MVR and the fact there were no dogs with congestive heart failure. Unaffected healthy CKCS would have been the ideal control group for assessment of the effects of MVR on markers of platelet activation. However, given the high prevalence of MVR in this breed, obtaining a large number of unaffected CKCS would have been difficult. The inclusion of dogs with congestive heart failure might have confounded the interpretation of results because several commonly used drugs, including diuretics and angiotensin-converting enzyme inhibitors, can reportedly affect platelet function in humans, (Kribben et al. 1988, Schäfer et al. 2003) and the same may be true in dogs.

In the present study, platelet activation, as assessed on the basis of MPC concentration and PCDW, was not a feature of subclinical CVHD in CKCS. Increased closure times in CKCS with a regurgitant jet size greater than 50% likely reflects quantitative and qualitative changes in VWF, as previously described (Tarnow et al. 2004). Significant differences in several platelet variables, including platelet count, MPV, analyser-derived PCT, MPC concentration, and PCDW, were detected between CKCS and dogs of other breeds. Such interbreed variation must be considered when interpreting results.

Further studies are required to investigate potential platelet activation in other breeds with CVHD as well as in other types of heart disease. It would

also be interesting to evaluate platelet activation in dogs with congestive heart failure (i.e. those most severely affected with turbulent high-velocity blood flow and fluid shear stress). Additional studies are indicated to identify the anticoagulant of choice for assessment of platelet indices such as MPC concentration, with the end goal to facilitate optimal clinical application. The identified interbreed variability in MPC concentration suggests that the requirement for breed-specific reference intervals should be investigated. Finally, the identified associations between markers of platelet activation (MPC concentration and PCDW) and platelet count warrant further research, particularly in animals with breed-associated thrombocytopenia.

6 References

Ahnadi, C., F. F. Boughrassa, E. S. Chapman-Montgomery, V. Poisson, A. Gervais, D. Okrongly, and A. M. Grant. 2004. "Comparison of two methods to assess variability of platelet response to anti-platelet therapies in patients with acute coronary syndrome undergoing angioplasty." *Thrombosis and Haemostasis* 92:1207-1213.

Ahnadi, C. E., E. S. Chapman, M. Lepine, D. Okrongly, N. Pujol-Moix, A. Hernandez, F. Boughrassa, and A. M. Grant. 2003. "Assessment of platelet activation in several different anticoagulants by the Advia 120 Hematology System, fluorescence flow cytometry, and electron microscopy." *Thrombosis and Haemostasis* 90 (5):940-948.

Arocha, F., M. Diez-Ewald, A. I. Durango, and T. Sulbarán. 1985. "Platelet activity in mitral valve prolapse: A study of platelet aggregation, malondialdehyde production, and plasma β-thromboglobulin." *American journal of hematology* 19 (1):21-25.

Atkins, C., J. Bonagura, S. Ettinger, P. Fox, S. Gordon, J. Häggström, R. Hamlin, B. Keene, V. Luis-Fuentes, and R. Stepien. 2009. "Guidelines for the diagnosis and treatment of canine chronic valvular heart disease." *Journal of Veterinary Internal Medicine* 23 (6):1142-1150.

Bae, S. H., J. Lee, K. H. Roh, and J. Kim. 2003. "Platelet activation in patients with diabetic retinopathy." *Korean Journal of Ophthalmology* 17 (2):140-144.

Barger, A. M. 2003. "The complete blood cell count: a powerful diagnostic tool." *The Veterinary clinics of North America. Small animal practice* 33 (6):1207-1222.

Barnett, H. M., M. W. Jones, D. R. Boughner, and W. J. Kostuk. 1976. "Cerebral ischemic events associated with prolapsing mitral valve." *Archives of Neurology* 33 (11):777-782.

Bauer, N. B., E. Er, and A. Moritz. 2012. "Effect of submaximal aerobic exercise on platelet function, platelet activation, and secondary and tertiary hemostasis in dogs." *American Journal of Veterinary Research* 73 (1):125-133.

Beardow, A. W., and J. W. Buchanan. 1993. "Chronic mitral-valve disease in Cavalier King Charles spaniels - 95 cases (1987-1991)." *Journal of the American Veterinary Medical Association* 203 (7):1023-1029.

Bertazzolo, W., S. Comazzi, L. Sesso, P. Scarpa, G. Ru, and S. Paltrinieri. 2007. "Comparison of methods for determining platelet numbers and volume in Cavalier King Charles spaniels." *Journal of Small Animal Practice* 48 (10):556-561.

Bizzaro, N. 1995. "EDTA-dependent pseudothrombocytopenia: A clinical and epidemiological study of 112 cases, with 10-year follow-up." *American journal of hematology* 50 (2):103-109.

Blann, A. D., S. K. Nadar, and G. Y. Lip. 2003. "The adhesion molecule P-selectin and cardiovascular disease." *European heart journal* 24 (24):2166-2179.

Bommer, N., D. Shaw, E. Milne, and A. Ridyard. 2008. "Platelet distribution width and mean platelet volume in the interpretation of thrombocytopenia in dogs." *Journal of Small Animal Practice* 49 (10):518-524.

Bonow, R. O., B. Carabello, A. C. de Leon, L. H. Edmunds, B. J. Fedderly, M. D. Freed, W. H. Gaasch, C. R. McKay, R. A. Nishimura, and P. T. O'Gara. 1998. "Guidelines for the Management of Patients With Valvular Heart Disease Executive Summary A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee on Management of Patients With Valvular Heart Disease)." *Circulation* 98 (18):1949-1984.

Borgarelli, M., P. Savarino, S. Crosara, R. Santilli, D. Chiavegato, M. Poggi, C. Bellino, G. La Rosa, R. Zanatta, and J. Häggström. 2008. "Survival characteristics and prognostic variables of dogs with mitral regurgitation attributable to myxomatous valve disease." *Journal of Veterinary Internal Medicine* 22 (1):120-128.

Boudreaux, M. K. 2008. "Characteristics, diagnosis, and treatment of inherited platelet disorders in mammals." *Journal of the American Veterinary Medical Association* 233 (8):1251-1259.

Boudreaux, M. K. 2010a. "Platelet structure." In *Schalm's veterinary haematology*, edited by D. J. Weiss and K.J. Wardrop, 561-568. Iowa, USA: Wiley-Blackwell.

Boudreaux, M. K. 2010b. "Platelets." In *Schalm's veterinary hematology*, edited by D.J Weiss and K. J Wardrop, 561-631. Blackwell Publishing.

Boudreaux, M. K. 2010c. "Thrombopoiesis." In *Schalm's Veterinary Haematology*, edited by D.J. Weiss and K.J. Wardrop, 56-60. Iowa, USA: Wiley-Blackwell.

Boudreaux, M. K., and J. L. Catalfamo. 2010. "Platelet biochemistry, signal transduction and function." In *Schalm's veterinary hematology*, edited by D. J. Weiss and K.J. Wardrop, 569-575. Iowa, USA: Blackwell Publishing Ltd.

Brass, L. F., S. T.J., L. Zhu, and D. S. Woulfe. 2013. "Signal transduction during platelet plug formation." In *Platelets*, edited by A.D. Michelson, 367-398. Amsterdam: Academic Press/Elsevier.

Briggs, C., P. Harrison, and S. Machin. 2007. "Continuing developments with the automated platelet count1." *International journal of laboratory hematology* 29 (2):77-91.

Brown, C., L. B. Leverett, C. W. Lewis, C. Alfrey, and J. Hellums. 1975. "Morphological, biochemical, and functional changes in human platelets subjected to shear stress." *J Lab Clin Med* 86 (3):462-471.

Brown, S. J., K. W. Simpson, S. Baker, M. A. Spagnoletti, and C. M. Elwood. 1994. "Macrothrombocytosis in Cavalier King Charles spaniels." *Veterinary Record* 135 (12):281-283.

Brummitt, D. R., and H. F. Barker. 2000. "The determination of a reference range for new platelet parameters produced by the Bayer ADVIA120 full blood count analyser." *Clinical and Laboratory Haematology* 22 (2):103-107.

Buchanan, J. W., A. W. Beardow, and C. D. Sammarco. 1997. "Femoral artery occlusion in Cavalier King Charles Spaniels." *Journal of the American Veterinary Medical Association* 211 (7):872-874.

Burke, A. P., and R. Virmani. 1998. "Intramural coronary artery dysplasia of the ventricular septum and sudden death." *Human pathology* 29 (10):1124-1127.

Butkiewicz, A. M., H. Kemona, V. Dymicka-Piekarska, J. Matowicka-Karna, P. Radziwon, and A. Lipska. 2006. "Platelet count, mean platelet volume and thrombocytopoietic indices in healthy women and men." *Thrombosis Research* 118 (2):199-204.

Callan, M. B., and U. Giger. 2001. "Assessment of a point-of-care instrument for identification of primary hemostatic disorders in dogs." *American journal of veterinary research* 62 (5):652-658.

Carr Jr, M. E. 1997. "In vitro assessment of platelet function." *Transfusion Medicine Reviews* 11 (2):106-115.

Casonato, A., A. Bertomoro, E. Pontara, D. Dannhauser, A. Lazzaro, and A. Girolami. 1994. "EDTA dependent pseudothrombocytopenia caused by antibodies against the cytoadhesive receptor of platelet gpIIB-IIIA." *Journal of clinical pathology* 47 (7):625-630.

Cay, N., A. Ipek, M. Gumus, Z. Birkan, and E. Ozmen. 2012. "Platelet activity indices in patients with deep vein thrombosis." *Clinical and Applied Thrombosis/Hemostasis* 18 (2):206-210.

Chapman, E. S., G. M. Colella, E. J. Hetherington, L. Paseltiner, and D. Zelmanovic. 1998. Automated method and device for identifying and quantifying platelets and for determining platelet activation state using whole blood samples. Google Patents.

Chapman, E. S., M. Sorette, E. Hetherington, D. Zelmanovic, G. Kling, J. Dugailliez, N. Pujol-Moix, and D. Okrongly. 2003. "A rapid, automated flow cytometric method to measure activated degranulated platelets by density determination." *Thrombosis and Haemostasis* 89 (6):1004-1015.

Chetboul, V., and R. Tissier. 2012. "Echocardiographic assessment of canine degenerative mitral valve disease." *Journal of Veterinary Cardiology* 14 (1):127-148.

Chetboul, V., R. Tissier, F. Villaret, A. Nicolle, E. Déan, T. Benalloul, and J.-L. Pouchelon. 2004. "Epidemiological, clinical, echo-doppler characteristics of mitral valve endocardiosis in Cavalier King Charles in France: a retrospective study of 451 cases (1995 to 2003)." *Canadian veterinary journal* 45 (12):1012-1015.

Christopherson, P. W., E. A. Spangler, and M. K. Boudreaux. 2012. "Evaluation and clinical application of platelet function testing in small animal practice." *Veterinary Clinics of North America: Small Animal Practice* 42 (1):173-188.

Chung, I., A. Choudhury, and G. Y. Lip. 2007. "Platelet activation in acute, decompensated congestive heart failure." *Thrombosis research* 120 (5):709-713.

Chung, I., and G. Y. Lip. 2006. "Platelets and heart failure." *European heart journal* 27 (22):2623-2631.

Clancey, N., S. Burton, B. Horney, A. MacKenzie, A. Nicastro, and E. Cote. 2009a. "Evaluation of platelet function in dogs with cardiac disease using the PFA-100 platelet function analyzer." *Veterinary Clinical Pathology* 38 (3):299-305.

Clancey, N., S. Burton, B. Horney, A. MacKenzie, A. Nicastro, and E. Côté. 2009b. "Effects of in vitro hemodilution of canine blood on platelet function analysis using the PFA-100." *Veterinary Clinical Pathology* 38 (4):467-470.

Clemmons, R., and K. Meyers. 1984. "Acquisition and aggregation of canine blood platelets: basic mechanisms of function and differences because of breed origin." *American journal of veterinary research* 45 (1):137.

Cornell, C. C., M. D. Kittleson, P. D. Torre, J. Häggström, C. W. Lombard, H. D. Pedersen, A. Vollmar, and A. Wey. 2004. "Allometric Scaling of M-Mode Cardiac Measurements in Normal Adult Dogs." *Journal of Veterinary Internal Medicine* 18 (3):311-321.

Cowan, S. M., J. W. Bartges, R. E. Gompf, J. R. Hayes, T. D. Moyers, C. C. Snider, D. A. Gerard, R. A. Craft, R. A. Muenchen, and R. C. Carroll. 2004. "Giant platelet disorder in the Cavalier King Charles Spaniel." *Experimental Hematology* 32 (4):344-350.

Cudillo, L., F. Laghi, R. Landolfi, G. Leone, S. Astore, S. Pelargonio, and B. Bizzi. 1983. "Beta-thromboglobulin and platelet regeneration time in children with mitral valve prolapse." *Giornale italiano di cardiologia* 13 (10):215-218.

Davies, M., B. Moore, and M. Braimbridge. 1978. "The floppy mitral valve. Study of incidence, pathology, and complications in surgical, necropsy, and forensic material." *British Heart Journal* 40 (5):468-481.

Davis, B., M. Toivio-Kinnucan, S. Schuller, and M. K. Boudreaux. 2008. "Mutation in beta 1-tubulin correlates with macrothrombocytopenia in cavalier king charles spaniels." *Journal of Veterinary Internal Medicine* 22 (3):540-545.

Devereux, R., W. Brown, R. Kramer-Fox, and I. Sachs. 1982. "Inheritance of mitral valve prolapse: effect of age and sex on gene expression." *Annals of internal medicine* 97 (6):826-832.

Dosh, K., and S. Steinhubl. 2010. "Monitoring of antithrombotic therapies in interventional cardiology." In *Antithrombotic Drug Therapy in Cardiovascular Disease*, edited by A. T. Askari and A. M. Lincoff, 307-330. New York, USA: Humana press.

Drachman, J. G. 2004. "Inherited thrombocytopenia: when a low platelet count does not mean ITP." *Blood* 103 (2):390-398.

Eksell, P., J. Häggström, C. Kvart, and A. Karlsson. 1994. "Thrombocytopenia in the Cavalier King Charles spaniel." *Journal of Small Animal Practice* 35 (3):153-155.

Falk, T., and L. Jönsson. 2000. "Ischaemic heart disease in the dog: a review of 65 cases." *Journal of Small Animal Practice* 41 (3):97-103.

Falk, T., L. Jönsson, L. H. Olsen, and H. D. Pedersen. 2006. "Arteriosclerotic changes in the myocardium, lung, and kidney in dogs with chronic congestive heart failure and myxomatous mitral valve disease." *Cardiovascular Pathology* 15 (4):185-193.

Falk, T., L. Jönsson, L. Olsen, I. Tarnow, and H. Ledersen. 2007. "Correlation of cardiac pathology and clinical findings in dogs with naturally occurring congestive heart failure." Proceedings of 17th Annual ECVIM Congress, Budapest.

Fiorin, F., A. Steffan, P. Pradella, N. Bizzaro, R. Potenza, and V. De Angelis. 1998. "IgG platelet antibodies in EDTA-dependent pseudothrombocytopenia bind to platelet membrane glycoprotein IIb." *American Journal of Clinical Pathology* 110 (2):178-183.

Fisher, M., B. Weiner, I. S. Ockene, A. Forsberg, C. P. Duffy, and P. H. Levine. 1983. "Platelet activation and mitral valve prolapse." *Neurology* 33 (3):384-384.

French, A. T., R. Ogden, C. Eland, G. Hemani, R. Pong-Wong, B. Corcoran, and K. M. Summers. 2012. "Genome-wide analysis of mitral valve disease in Cavalier King Charles Spaniels." *Veterinary journal* 193 (1):283-286.

Fuse, S., and T. Kamiya. 1994. "Plasma thromboxane B2 concentration in pulmonary hypertension associated with congenital heart disease." *Circulation* 90 (6):2952-2955.

Gaal, T., D. Halmay, R. Kocsis, and Z. Abonyi-Tóth. 2007. "Evaluation of the effect of ketoprofen and carprofen on platelet function in dogs studied by PFA-100 point-of-care analyser." *Acta Veterinaria Hungarica* 55 (3):287-294.

Gelain, M. E., G. F. Tutino, E. Pogliani, and W. Bertazzolo. 2010. "Macrothrombocytopenia in a group of related Norfolk terriers." *Veterinary Record* 167 (13):493-4.

Giacomini, A., P. Legovini, G. Gessoni, F. Antico, S. Valverde, M. Salvadego, and F. Manoni. 2001. "Platelet count and parameters determined by the Bayer ADVIATM 120 in reference subjects and patients." *Clinical & Laboratory Haematology* 23 (3):181-186.

Gibbs, C. R., A. D. Blann, R. D. Watson, and G. Y. Lip. 2001. "Abnormalities of Hemorheological, Endothelial, and Platelet Function in Patients With Chronic Heart Failure in Sinus Rhythm Effects of Angiotensin-Converting Enzyme Inhibitor and β-Blocker Therapy." *Circulation* 103 (13):1746-1751.

Goldsmith, I., A. Blann, R. Patel, and G. Lip. 2000. "Plasma fibrinogen, soluble P-selectin, and von Willebrand factor in aortic valve disease: evidence for abnormal haemorheology, platelet activation, and endothelial dysfunction." *Heart* 83 (5):577-578.

Gouni, V., F. J. Serres, J.-L. Pouchelon, R. Tissier, H. P. Lefebvre, A. P. Nicolle, C. C. Sampedrano, and V. Chetboul. 2007. "Quantification of mitral valve regurgitation in dogs with degenerative mitral valve disease by use of the proximal isovelocity surface area method." *Journal of the American Veterinary Medical Association* 231 (3):399-406.

Häggström, J., A. Boswood, M. O'Grady, O. Jons, S. Smith, S. Swift, M. Borgarelli, B. Gavaghan, J. G. Kresken, and M. Patteson. 2008. "Effect of pimobendan or benazepril hydrochloride on survival times in dogs with congestive heart failure caused by naturally occurring myxomatous mitral valve disease: the QUEST study." *Journal of Veterinary Internal Medicine* 22 (5):1124-1135.

Häggström, J., K. Hansson, C. Kvart, and L. Swenson. 1992. "Chronic valvular disease in the Cavalier King Charles spaniel in Sweden." *Veterinary Record* 131 (24):549-553.

Häggström, J., C. Kvart, and K. Hansson. 1995. "Heart sounds and murmurs - changes related to severity of chronic valvular disease in the Cavalier King Charles spaniel." *Journal of Veterinary Internal Medicine* 9 (2):75-85.

Häggström, J., and H. D. Pedersen. 2005. "Acquired valvular heart disease." In *Textbook of Veterinary Internal Medicine*, edited by S. J. Ettinger and E. C. Feldman, 1022-1039. St Louis: Elsevier.

Handagama, P., B. Feldman, C. Kono, and T. Farver. 1986. "Mean platelet volume artifacts: the effect of anticoagulants and temperature on canine platelets." *Veterinary Clinical Pathology* 15 (4):13-17.

Hansson, K., J. Häggström, C. Kvart, and P. Lord. 2002. "Left atrial to aortic root indices using two-dimensional and M-mode echocardiography in Cavalier King Charles spaniels with and without left atrial enlargement." *Veterinary Radiology & Ultrasound* 43 (6):568-575.

Harrison, P., and C. Briggs. 2013. "Platelet counting." In *Platelets*, edited by A.D Michelson, 547-557. Third edition. Academic Press.

Harrison, P., A. Horton, D. Grant, C. Briggs, and S. Machin. 2000. "Immunoplatelet counting: a proposed new reference procedure." *British journal of haematology* 108 (2):228-235.

Hartert, H. 1948. "Blutgerinnungsstudien mit der Thrombelastographie; einem neuen Untersuchungs verfahren [Coagulation analysis with thrombelastography, a new method]." *Klin Wochenschr* 26:577-583.

Hartert, H. 1951. "Die thrombelastographie. Eine methode zur physikalischen analyse des blutgerinnungsvarganges [Thrombelastography, a method for physical analysis of blood coagulation]." *Z Gesamte Exp Med* 117:189-203.

Hinchcliff, K., G. Kociba, and L. Mitten. 1993. "Diagnosis of EDTA-dependent pseudothrombocytopenia in a horse." *Journal of the American Veterinary Medical Association* 203 (12):1715.

Hoffman, M. 2001. "A cell-based model of hemostasis." *Thromb Haemost* 85:958-965.

Holme, S., M. Simmonds, R. Ballek, and S. Murphy. 1981. "Comparative measurements of platelet size by Coulter Counter, microscopy of blood smears, and light-transmission studies. Relationship between platelet size and shape." *The Journal of laboratory and clinical medicine* 97 (5):610-622.

Icli, A., F. Aksoy, A. Dogan, A. Arslan, I. Ersoy, H. Yucel, and O. Gorgulu. 2013. "Mean platelet volume may be elevated in mitral valve prolapse and associated with the severity of prolapse." *Clinical and Applied Thrombosis/Hemostasis* 19:608-612.

Italiano, J. E., and J. H. Hartwig. 2013. "Megakaryocyte development and platelet formation." In *Platelets*, edited by A.D. Michelson, 27-49. Amsterdam: Academic Press.

Jandrey, K. E. 2012. "Assessment of platelet function." *Journal of Veterinary Emergency and Critical Care* 22 (1):81-98.

Jönsson, L. 1972. "Coronary arterial lesions and myocardial infarcts in the dog. A pathologic and microangiographic study." *Acta veterinaria Scandinavica. Supplementum* 38:1-80.

Kaito, K., H. Otsubo, N. Usui, M. Yoshida, J. Tanno, E. Kurihara, K. Matsumoto, R. Hirata, K. Domitsu, and M. Kobayashi. 2005. "Platelet size deviation width, platelet large cell ratio, and mean platelet volume have sufficient sensitivity and specificity in the diagnosis of immune thrombocytopenia." *British journal of haematology* 128 (5):698-702.

Kamath, S., A. Blann, and G. Lip. 2001. "Platelet activation: assessment and quantification." *European heart journal* 22 (17):1561-1571.

Karlstam, E., J. Häggström, C. Kvart, L. Jönsson, and M. Michaelsson. 2000. "Pulmonary artery lesions in Cavalier King Charles spaniels." *Veterinary Record* 147 (6):166-167.

Keidel, A., and R. Mischke. 1998. "Clinical evaluation of platelet function analyzer PFA-100 in dogs." *Berliner Und Munchener Tierarztliche Wochenschrift* 111 (11-12):452-456.

Kelley, J., L. C. Sharkey, P. W. Christopherson, and A. Rendahl. 2014. "Platelet count and plateletcrit in Cavalier King Charles Spaniels and Greyhounds using the Advia 120 and 2120." *Veterinary Clinical Pathology* 43 (1):43-49.

Kelley, J. A. 2013. "Platelet count and plateletcrit in Cavalier King Charles Spaniels and Greyhounds using the Advia 120/2120 (Masters thesis)." University of Minnesota.

Kennon, S., C. Price, P. Mills, M. Macey, J. Cooper, H. Clarke, and A. Timmis. 2003. "The central role of platelet activation in determining the severity of acute coronary syndromes." *Heart* 89 (10):1253-1254.

Kittleson, M. D., and W. A. Brown. 2003. "Regurgitant fraction measured by using the proximal isovelocity surface area method in dogs with chronic myxomatous mitral valve disease." *Journal of veterinary internal medicine* 17 (1):84-88.

Kogure, K. 1980. "Pathology of chronic mitral valvular disease in the dog." *Japanese Journal of Veterinary Science* 42 (3):323-335.

Koide, M., M. Hamawaki, T. Narishige, H. Sato, S. Nemoto, G. DeFreyte, M. R. Zile, G. Cooper IV, and B. A. Carabello. 2000. "Microtubule depolymerization normalizes in vivo myocardial contractile function in dogs with pressure-overload left ventricular hypertrophy." *Circulation* 102 (9):1045-1052.

Kol, A., and D. L. Borjesson. 2010. "Application of thrombelastography/thromboelastometry to veterinary medicine." *Veterinary Clinical Pathology* 39 (4):405-416.

Koplitz, S. L., M. A. Scott, and L. A. Cohn. 2001. "Effects of platelet clumping on platelet concentrations measured by use of impedance or buffy coat analysis in dogs." *Journal of the American Veterinary Medical Association* 219 (11):1552-1556.

Kostuk, W., D. Boughner, H. Barnett, and M. Silver. 1977. "Strokes: a complication of mitral-leaflet prolapse?" *The Lancet* 310 (8033):313-316.

Kratz, A., M. J. Wood, A. J. Siegel, J. R. Hiers, and E. M. Van Cott. 2006. "Effects of Marathon Running on Platelet Activation Markers Direct Evidence for In Vivo Platelet Activation." *American journal of clinical pathology* 125 (2):296-300.

Kribben, A., E. Fritschka, M. Sibold, M. Fassbender, M. Rothschild, A. Distler, and T. Philipp. 1988. "Different effects of furosemide on alpha-adrenoceptors and on platelet aggregation in man." *Klinische Wochenschrift* 66 (14):624-627.

Kuter, D. J. 1996. "The physiology of platelet production." *Stem Cells* 14 (S1):88-101.

Kvart, C., J. Häggström, H. D. Pedersen, K. Hansson, A. Eriksson, A. K. Järvinen, A. Tidholm, K. Bsenko, E. Ahlgren, and M. Lives. 2002. "Efficacy of enalapril for prevention of congestive heart failure in dogs with myxomatous valve disease and asymptomatic mitral regurgitation." *Journal of veterinary internal medicine* 16 (1):80-88.

Lancé, M. D., M. Sloep, Y. M. Henskens, and M. A. Marcus. 2012. "Mean Platelet Volume as a Diagnostic Marker for Cardiovascular Disease Drawbacks of Preanalytical Conditions and Measuring Techniques." *Clinical and applied thrombosis/hemostasis* 18 (6):561-568.

Leandro-García, L. J., S. Leskelä, I. Landa, C. Montero-Conde, E. López-Jiménez, R. Letón, A. Cascón, M. Robledo, and C. Rodríguez-Antona. 2010. "Tumoral and tissue-specific expression of the major human β-tubulin isotypes." *Cytoskeleton* 67 (4):214-223.

Lewis, T., S. Swift, J. A. Woolliams, and S. Blott. 2011. "Heritability of premature mitral valve disease in Cavalier King Charles spaniels." *Veterinary Journal* 188 (1):73-76.

Lilliehöök, I., and H. Tvedten. 2009. "Validation of the Sysmex XT-2000iV hematology system for dogs, cats, and horses. I. Erythrocytes, platelets, and total leukocyte counts." *Veterinary clinical pathology* 38 (2):163-174.

Lin, S., M. Fisher, T. Tak, S. Rahimtoola, and P. Chandraratna. 1989. "Platelet activation in patients with mitral valve prolapse." *The Canadian journal of cardiology* 5 (2):84.

Ljungvall, I., C. Ahlstrom, K. Hoglund, P. Hult, C. Kvart, M. Borgarelli, P. Ask, and J. Häggström. 2009. "Use of signal analysis of heart sounds and murmurs to assess severity of mitral valve regurgitation attributable to myxomatous mitral valve disease in dogs." *American journal of veterinary research* 70 (5):604-613.

Lord, P., K. Hansson, C. Kvart, and J. Häggström. 2010. "Rate of change of heart size before congestive heart failure in dogs with mitral regurgitation." *Journal of Small Animal Practice* 51 (4):210-218.

Macey, M., U. Azam, D. McCarthy, L. Webb, E. S. Chapman, D. Okrongly, D. Zelmanovic, and A. Newland. 2002. "Evaluation of the anticoagulants EDTA and citrate, theophylline, adenosine, and dipyridamole (CTAD) for assessing platelet activation on the ADVIA 120 Hematology System." *Clinical Chemistry* 48 (6):891-899.

Macey, M., E. Carty, L. Webb, E. Chapman, D. Zelmanovic, D. Okrongly, D. Rampton, and A. Newland. 1999. "Use of mean platelet component to measure platelet activation on the ADVIA 120 haematology system." *Cytometry* 38 (5):250-255.

Madsen, M. B., L. H. Olsen, J. Häggström, K. Hoglund, I. Ljungvall, T. Falk, G. Wess, H. Stephenson, J. Dukes-McEwan, V. Chetboul, V. Gouni, H. F. Proschowsky, S. Cirera, P. Karlskov-Mortensen, and M. Fredholm. 2011. "Identification of 2 Loci Associated with Development of Myxomatous Mitral Valve Disease in Cavalier King Charles Spaniels." *Journal of Heredity* 102:S62-S67.

Martini, F., A. Zuppiroli, A. M. Gori, E. Chiarantini, S. Fedi, D. Prisco, A. P. Cellai, V. Boddi, R. Abbate, A. Dolara, and G. F. Gensini. 1996. "Platelet and blood clotting activation in patients with mitral valve prolapse." *Thrombosis Research* 83 (4):299-306.

McNiel, E. A., G. K. Ogilvie, M. J. Fettman, and M. D. Salman. 1997. "Platelet hyperfunction in dogs with malignancies." *Journal of Veterinary Internal Medicine* 11 (3):178-182.

Michelson, A. D. 2004. "Platelet function testing in cardiovascular diseases." *Circulation* 110 (19):e489-e493.

Mischke, R., and A. Keidel. 2002. "Preclinical studies for the use of the platelet function analyser PFA-100 (collagen/ADP cartridge) in dogs." *Deutsche tierarztliche wochenschrift* 109 (5):235-238.

Mischke, R., and A. Keidel. 2003. "Influence of platelet count, acetylsalicylic acid, von Willebrand's disease, coagulopathies, and haematocrit on results obtained using a platelet function analyser in dogs." *Veterinary journal* 165 (1):43-52.

Moesgaard, S. G., T. M. Sorensen, A. Sterup, I. Tarnow, A. T. Kristensen, A. L. Jensen, and L. H. Olsen. 2009. "Changes in platelet function in Dachshunds with early stages of myxomatous mitral valve disease." *Research in Veterinary Science* 86 (2):320-324.

Moritz, A., and M. Becker. 2010. "Automated hematology systems." In *Schalm's Veterinary hematology*, edited by B. Feldman, J. G. Zinkl and N. C. Jain, 1054-1066. Blackwell Publishing.

Moritz, A., Y. Fickenscher, K. Meyer, K. Failing, and D. J. Weiss. 2004. "Canine and feline hematology reference values for the ADVIA 120 hematology system." *Veterinary Clinical Pathology* 33 (1):32-38.

Moritz, A., B. K. Walcheck, J. Deye, and D. J. Weiss. 2003. "Effects of short-term racing activity on platelet and neutrophil activation in dogs." *American journal of veterinary research* 64 (7):855-859.

Moritz, A., B. K. Walcheck, and D. J. Weiss. 2005. "Evaluation of flow cytometric and automated methods for detection of activated platelets in dogs with inflammatory disease." *American Journal of Veterinary Research* 66 (2):325-329.

Muzzi, R. A., R. B. de Araújo, L. A. Muzzi, J. L. Pena, and E. F. Silva. 2003. "Regurgitant jet area by Doppler color flow mapping: quantitative assessment of mitral regurgitation severity in dogs." *Journal of Veterinary Cardiology* 5 (2):33-38.

Nielsen, L. A., N. E. Zois, H. D. Pedersen, L. H. Olsen, and I. Tarnow. 2007. "Platelet function in dogs: breed differences and effect of acetylsalicylic acid administration." *Veterinary Clinical Pathology* 36 (3):267-273.

Northern, J., and H. Tvedten. 1992. "Diagnosis of microthrombocytosis and immune-mediated thrombocytopenia in dogs with thrombocytopenia: 68 cases (1987–1989)." *J Am Vet Med Assoc* 200 (3):368-372.

O'Connor, C. M., P. A. Gurbel, and V. L. Serebruany. 1999. "Usefulness of soluble and surface-bound P-selectin in detecting heightened platelet activity in patients with congestive heart failure." *The American journal of cardiology* 83 (9):1345-1349.

O'Gara, P., L. Sugeng, R. Lang, M. Sarano, J. Hung, S. Raman, G. Fischer, B. Carabello, D. Adams, and M. Vannan. 2008. "The role of imaging in chronic degenerative mitral regurgitation." *JACC: Cardiovascular Imaging* 1 (2):221-237.

Olsen, L. H., M. Fredholm, and H. D. Pedersen. 1999. "Epidemiology and inheritance of mitral valve prolapse in Dachshunds." *Journal of Veterinary Internal Medicine* 13 (5):448-456.

Olsen, L. H., A. T. Kristensen, J. Häggström, A. L. Jensen, B. Klitgaard, H. Hansson, and H. D. Pedersen. 2001. "Increased platelet aggregation response in cavalier king charles spaniels with mitral valve prolapse." *Journal of Veterinary Internal Medicine* 15 (3):209-216.

Olsen, L. H., A. T. Kristensen, K. Qvortrup, and H. D. Pedersen. 2004. "Comparison of manual and automated methods for determining platelet counts in dogs with macrothrombocytopenia." *Journal of Veterinary Diagnostic Investigation* 16 (2):167-170.

Onder, O., A. Weinstein, and L. W. Hoyer. 1980. "Pseudothrombocytopenia caused by platelet agglutinins that are reactive in blood anticoagulated with chelating agents." *Blood* 56 (2):177-182.

Oyama, M. A., and S. V. Chittur. 2006. "Genomic expression patterns of mitral valve tissues from dogs with degenerative mitral valve disease." *American Journal of Veterinary Research* 67 (8):1307-1318.

Park, Y., N. Schoene, and W. Harris. 2002. "Mean platelet volume as an indicator of platelet activation: methodological issues." *Platelets* 13 (5-6):301-306.

Patterson, D., D. Detweiler, K. Hubben, and R. Botts. 1961. "Spontaneous abnormal cardiac arrhythmias and conduction disturbances in the dog. A clinical and pathologic study of 3,000 dogs." *American Journal of Veterinary Research* 22:355-369.

Pawlus, J., M. Hołub, M. Kożuch, M. Dąbrowska, and S. Dobrzycki. 2010. "Serum myeloperoxidase levels and platelet activation parameters as diagnostic and prognostic markers in the course of coronary disease." *International journal of laboratory hematology* 32 (3):320-328.

Payne, B. 1985. "EDTA-induced pseudothrombocytopenia. Recognizing a laboratory artifact." *Postgraduate medicine* 77 (8):75-76.

Pedersen, D., K. Lorentzen, and B. Kristensen. 1999a. "Echocardiographic mitral valve prolapse in Cavalier King Charles spaniels: epidemiology and prognostic significance for regurgitation." *Veterinary record* 144 (12):315-320.

Pedersen, H., B. Nørby, and K. Lorentzen. 1996. "Echocardiographic study of mitral valve prolapse in Dachshunds." *Journal of Veterinary Medicine Series A* 43 (1-10):103-110.

Pedersen, H. D., and J. Häggström. 2000. "Mitral valve prolapse in the dog: a model of mitral valve prolapse in man." *Cardiovascular research* 47 (2):234-243.

Pedersen, H. D., J. Häggström, T. Falk, T. Mow, L. H. Olsen, L. Iversen, and A. L. Jensen. 1999b. "Auscultation in mild mitral regurgitation in dogs: observer variation, effects of physical maneuvers, and agreement with color Doppler echocardiography and phonocardiography." *Journal of Veterinary Internal Medicine* 13 (1):56-64.

Pedersen, H. D., J. Häggström, L. H. Olsen, K. Christensen, A. Selin, M. L. Burmeister, and H. Larsen. 2002. "Idiopathic asymptomatic thrombocytopenia in Cavalier King Charles Spaniels is an autosomal recessive trait." *Journal of Veterinary Internal Medicine* 16 (2):169-173.

Pedersen, H. D., B. O. Kristensen, K. A. Lorentzen, J. Koch, A. L. Jensen, and A. Flagstad. 1995. "Mitral valve prolapse in 3-year-old healthy Cavalier King Charles Spaniels. An echocardiographic study." *Canadian Journal of Veterinary Research* 59 (4):294-298.

Pewarchuk, W., J. VanderBoom, and M. Blajchman. 1992. "Pseudopolycythemia, pseudothrombocytopenia, and pseudoleukopenia due to overfilling of blood collection vacuum tubes." *Archives of pathology & laboratory medicine* 116 (1):90-92.

Pomerance, A., and J. C. Whitney. 1970. "Heart valve changes common to man and dog: a comparative study." *Cardiovasc. Res.* 4:61-66.

Ragan, H. 1972. "Platelet agglutination induced by ethylenediaminetetraacetic acid in blood samples from a miniature pig." *American Journal of Veterinary Research* 33 (12):2601-2603.

Rizzi, T. E., J. H. Meinkoth, and K. D. Clinkenbeard. 2010. "Normal hematology of the dog." In *Schalm's veterinary hematology*, edited by D.J Weiss and J Wardrop, 799-810. Iowa, USA: Wiley Blackwell.

Russell, K. G. 2010. "Platelet kinetics and laboratory evaluation of thrombocytopenia." In *Schalm's veterinary haematology* edited by D. J. Weiss and K.J. Wardrop, 576-585. Iowa, USA: Blackwell.

Sahn, D. J., A. DeMaria, A. Kisslo, and A. Weyman. 1978. "Recommendation regarding quantification in M-mode echocardiography: results of a survey of echocardiographic measurements." *Circulation* 58:1072-1083.

Santoro, S. K., L. D. Garrett, and M. Wilkerson. 2007. "Platelet Concentrations and Platelet-Associated IgG in Greyhounds." *Journal of Veterinary Internal Medicine* 21 (1):107-112.

Sargent, J., D. J. Connolly, V. Watts, P. Motskula, H. A. Volk, C. R. Lamb, and L. Fuentes. 2015. "Assessment of mitral regurgitation in dogs: comparison of results of echocardiography with magentic resonance imaging." *Journal Small Anim Practice* 56 (11):641-650.

Schäfer, A., D. Fraccarollo, S. Hildemann, M. Christ, M. Eigenthaler, A. Kobsar, U. Walter, and J. Bauersachs. 2003. "Inhibition of platelet activation in congestive heart failure by aldosterone receptor antagonism and ACE inhibition." *Thrombosis and haemostasis* 89 (6):1024-1030.

Scharf, R., M. Hennerici, V. Bluschke, J. Lueck, and R. Kladetzky. 1982. "Cerebral ischemia in young patients: it is associated with mitral valve prolapse and abnormal platelet activity in vivo?" *Stroke* 13 (4):454-458.

Schutte, J. E., F. A. Gaffney, L. Blend, and C. G. Blomqvist. 1981. "Distinctive anthropometric characteristics of women with mitral valve prolapse." *The American journal of medicine* 71 (4):533-538.

Schwer, H. D., P. Lecine, S. Tiwari, J. E. Italiano, J. H. Hartwig, and R. A. Shivdasani. 2001. "A lineage-restricted and divergent β-tubulin isoform is essential for the biogenesis, structure and function of blood platelets." *Current Biology* 11 (8):579-586.

Shreiner, D. P., and W. R. Bell. 1973. "Pseudothrombocytopenia: manifestation of a new type of platelet agglutinin." *Blood* 42 (4):541-549.

Siemens. 2002. Advia 120 Haematology system operator's guide.

Singh, M. K., and W. A. Lamb. 2005. "Idiopathic thrombocytopenia in Cavalier King Charles Spaniels." *Australian Veterinary Journal* 83 (11):700-703.

Sisson, D., C. Kvart, and P. G. G. Darke. 1999. "Acquired valvular heart disease in dogs and cats (second edition)." In *Textbook of Canine and Feline Cardiology. Philadelphia: WB Saunders*, edited by P.R. Fox, D.D. Sisson and N.S. Moise, 536-566.

Smedile, L. E., D. M. Houston, S. M. Taylor, K. Post, and G. P. Searcy. 1997. "Idiopathic, asymptomatic thrombocytopenia in Cavalier King Charles Spaniels: 11 cases (1983-1993)." *Journal of the American Animal Hospital Association* 33 (5):411-415.

Smith, J. R., K. F. Smith, and B. M. Brainard. 2014. "Platelet parameters from an automated hematology analyzer in dogs with inflammatory clinical diseases." *The Veterinary Journal* 201 (3):406-411.

Stein, P. D., and H. N. Sabbah. 1976. "Turbulent blood flow in the ascending aorta of humans with normal and diseased aortic valves." *Circulation research* 39 (1):58-65.

Stokol, T., and H. N. Erb. 2007. "A comparison of platelet parameters in EDTA-and citrate-anticoagulated blood in dogs." *Veterinary Clinical Pathology* 36 (2):148-154.

Swenson, L., J. Häggström, C. Kvart, and R. K. Juneja. 1996. "Relationship between parental cardiac status in Cavalier King Charles Spaniels and prevalence and severity of chronic valvular disease in offspring." *Journal of the American Veterinary Medical Association* 208 (12):2009-2012.

Tanaka, R., A. Murota, Y. Nagashima, and Y. Yamane. 2002. "Changes in platelet life span in dogs with mitral valve regurgitation." *Journal of Veterinary Internal Medicine* 16 (4):446-451.

Tanaka, R., and Y. Yamane. 2000. "Platelet aggregation in dogs with mitral valve regurgitation." *American Journal of Veterinary Research* 61 (10):1248-1251.

Tarnow, I., and A. T. Kristensen. 2010. "Evaluation of platelet function." In *Schalm's veterinary haematology*, edited by D.J. Weiss and K.J. Wardrop, 1123-1131. Iowa, USA: Blackwell Publishing.

Tarnow, I., A. T. Kristensen, L. H. Olsen, T. Falk, L. Haubro, L. G. Pedersen, and H. D. Pedersen. 2005. "Dogs with Heart Diseases Causing Turbulent High-Velocity Blood Flow Have Changes in Platelet Function and von Willebrand Factor Multimer Distribution." *Journal of Veterinary Internal Medicine* 19 (4):515-522.

Tarnow, I., A. T. Kristensen, L. H. Olsen, and H. D. Pedersen. 2004. "Assessment of changes in hemostatic markers in Cavalier King Charles Spaniels with myxomatous mitral valve disease." *American Journal of Veterinary Research* 65 (12):1644-1652.

Tarnow, I., A. T. Kristensen, H. Texel, L. H. Olsen, and H. D. Pedersen. 2003. "Decreased platelet function in Cavalier King Charles spaniels with mitral valve regurgitation." *Journal of Veterinary Internal Medicine* 17 (5):680-686.

Tarnow, I., L. Olsen, S. Andreasen, S. Moesgaard, C. Rasmussen, A. Kristensen, and T. Falk. 2010a. "Congestive heart failure in dogs is associated with enhanced platelet-leukocyte aggregates-a marker for platelet activation." *Journal of Veterinary Internal Medicine* 24 (3):673-673.

Tarnow, I., L. Olsen, S. Moesgaard, A. Martinsen, A. Birkegaard, A. Kristensen, and B. Wiinberg. 2010b. "Thromboelastography in dogs with asymptomatic myxomatous mitral valve disease." *Journal of Veterinary Internal Medicine* 24 (3):673-673.

Thomas, W. P., C. E. Gaber, G. J. Jacobs, P. M. Kaplan, C. W. Lombard, N. Moise, and B. L. Moses. 1993. "Recommendations for Standards in Transthoracic Two-Dimensional Echocardiography in the Dog and Cat." *Journal of Veterinary Internal Medicine* 7 (4):247-252.

Thompson, C. B., and J. A. Jakubowski. 1988. "the patho-physiology and clinical relevance of platelet heterogeneity." *Blood* 72 (1):1-8.

Tilley, L. P., F. W. Smith, and B. Keene. 2006. Murmurs. In: Rapid Interpretation of Heart Sounds, Murmurs, and Arrhythmias: A Guide to Cardiac Auscultation in the Dog and Cat. St Louis, Mo: Saunders Elsevier.

Tse, H. F., C. P. Lau, and G. Cheng. 1997. "Relation between mitral regurgitation and platelet activation." *Journal of the American College of Cardiology* 30 (7):1813-1818.

Tsutsui, H., K. Ishihara, and G. Cooper. 1993. "Cytoskeletal role in the contractile dysfunction of hypertrophied myocardium." *Science* 260 (5108):682-687.

Tvedten, H. 2012. "Hemostatic abnormalities." In *Small animal clinical diagnosis by laboratory methods* edited by M.D. Willard and H. Tvedten, 92-111. Saunders.

Tvedten, H., I. Lilliehook, A. Hillstrom, and J. Häggström. 2008. "Plateletcrit is superior to platelet count for assessing platelet status in Cavalier King Charles Spaniels." *Veterinary Clinical Pathology* 37 (3):266-271.

Tvedten, H. W., I. E. Lilliehook, J. Oberg, J. Häggström, K. Hoglund, and I. Ljungvall. 2012. "Validation of Advia plateletcrit for assessing platelet mass in dogs, including Cavalier King Charles Spaniels." *Veterinary Clinical Pathology* 41 (3):336-343.

Varol, E., M. Ozaydin, Y. Türker, and S. Alaca. 2009. "Mean platelet volume, an indicator of platelet activation, is increased in patients with mitral stenosis and sinus rhythm." *Scandinavian journal of clinical and laboratory investigation* 69 (6):708-712.

Walsh, P., T. Kansu, J. Corbett, P. Savion, W. Goldburgh, and N. Schatz. 1981. "Platelets, thromboembolism and mitral valve prolapse." *Circulation* 63 (3):552-559.

White, J. G. 2013. "Platelet structure." In *Platelets*, edited by A.D. Michelson, 117-144. Amsterdam: Academic Press/Elsevier.

Whitney, J. 1974. "Observations on the effect of age on the severity of heart valve lesions in the dog." *Journal of Small Animal Practice* 15 (8):511-522.

Whitney, J. 1976. "Some aspects of the pathogenesis of canine arteriosclerosis." *Journal of Small Animal Practice* 17 (2):87-97.

Wilcken, D. E. 1992. "Genes, gender and geometry and the prolapsing mitral valve." *Australian and New Zealand journal of medicine* 22 (5):556-561.

Wills, T. B., and K. J. Wardrop. 2008. "Pseudothrombocytopenia secondary to the effects of EDTA in a dog." *Journal of the American Animal Hospital Association* 44 (2):95-97.

Wiwanitkit, V. 2004. "Plateletcrit, mean platelet volume, platelet distribution width: its expected values and correlation with parallel red blood cell parameters." *Clinical and applied thrombosis/hemostasis* 10 (2):175-178.

Yin, W.-H., J.-W. Chen, H.-L. Jen, M.-C. Chiang, W.-P. Huang, A.-N. Feng, S.-J. Lin, and M. S. Young. 2003. "The prognostic value of circulating soluble cell adhesion molecules in patients with chronic congestive heart failure." *European journal of heart failure* 5 (4):507-516.

Zelmanovic, D. 2006. "Interpretation of changes in PDW and MPC in stored canine blood." *Veterinary Clinical Pathology* 35 (2):140; author reply 140.

Zelmanovic, D., and E. J. Hetherington. 1998. "Automated Analysis of Feline Platelets in Whole Blood, Including Platelet Count, Mean Platelet Volume, and Activation State." *Veterinary Clinical Pathology* 27 (1):2-9.

Zoghbi, W. A., M. Enriquez-Sarano, E. Foster, P. A. Grayburn, C. D. Kraft, R. A. Levine, P. Nihoyannopoulos, C. M. Otto, M. A. Quinones, and H. Rakowski. 2003. "Recommendations for evaluation of the severity of native valvular regurgitation with two-dimensional and Doppler echocardiography." *Journal of the American Society of Echocardiography* 16 (7):777-802.

7 Appendices

7.1 Appendix 1: CKCS Signalment and auscultation findings

Dog	Case number	Bwt (kg)	Sex	Age (Years)	Murmur grade
1	3294.11	10.9	MN	11	5
2	482.12	6.6	MN	5	1
3	489.12	7.9	MN	3	1
4	580.12	11.6	MN	2	0
5	601.12	11.2	FE	10	3
6	600.12	14.56	MN	7	3
7	599.12	11.9	FN	8	2
8	709.12	12.76	MN	2	0
9	712.12	9.91	MN	2.5	0
10	716.12	10.1	FN	5.5	1
11	909.12	8.09	FE	6	4
12	911.12	12.7	FN	6	1
13	912.12	13.9	FN	8.5	1
14	913.12	7.5	MN	0.8	1
15	924.12	13.5	FN	10.5	4
16	994.12	7.1	FN	5	0
17	996.12	14.6	FN	7	1
18	995.12	5.5	MN	0.6	0
19	999.12	10.2	MN	3	0
20	1000.12	12.8	FE	3.5	0
21	1003.12	7.5	MN	0.8	1
22	1001.12	10.9	FN	4	0
23	1047.12	8.1	MN	15	5
24	1093.12	10.5	MN	9.3	4
25	1100.12	10.2	FN	4	0
26	1101.12	11.8	FN	4	0
27	1099.12	17.5	MN	2	0
28	1134.12	10.3	FN	6	1
29	1132.12	9.3	FN	3	1
30	1137.12	7.1	FE	3.5	0
31	1138.12	11.2	MN	4.5	2
32	1163.12	11.3	FN	3	2
33	1162.12	6.3	FE	1.3	0
34	1167.12	10.3	MN	5.5	3
35	1165.12	8.9	MN	4.5	0
36	1164.12	6.1	MN	0.83	0
37	1166.12	6.3	FN	3.5	0
38	1190.12	8.3	FN	9	2
39	1193.12	8.2	MN	6.8	0
40	1194.12	8.8	FN	6	1
41	1215.12	11	MN	2.25	0
42	1211.12	18.2	FN	4.5	1
43	1220.12	7.2	MN	1	1
44	1223.12	6.5	MN	0.7	1
45	1222.12	8.9	FN	3.5	2
46	1228.12	10.9	MN	5	0
47	1274.12	11.6	MN	2	0

Dog	Case number	Bwt (kg)	Sex	Age (Years)	Murmur grade
48	1273.12	15.7	MN	5.5	2
49	1394.12	11.35	MN	7	1
50	1479.12	8.2	ME	6	3
51	1478.12	8.1	FE	2	3
52	1476.12	8.5	MN	13	4
53	1477.12	8.8	FE	4	1
54	1480.12	11.2	FN	3	0
55	1483.12	6.2	FN	4	2
56	1546.12	9.4	ME	8.5	3
57	1583.12	8.8	FN	13	3
58	1576.12	5.2	FN	1	2
59	1590.12	10.7	MN	3.5	1
60	1591.12	10.9	MN	2.5	1
61	1589.12	7	FN	0.8	0
62	1694.12	8.2	MN	3	0
63	1697.12	10.8	MN	2	1
64	1797.12	5.89	FN	1	0
65	1799.12	8.5	MN	1	2
66	1886.12	10.9	MN	13	0
67	1902.12	9.3	FN	5	0
68	1885.12	11.9	FN	6	0
69	2001.12	7.7	FN	6	3
70	1997.12	9.8	MN	6	0
71	2003.12	7.15	MN	0.7	0
72	2292.12	9.83	FN	1	0
73	2293.12	9.9	MN	11	4
74	2439.12	12.4	FN	6	2
75	2433.12	7.8	FN	6	2
76	4194.12	8.7	ME	1.2	0
77	4196.12	8.9	FE	1.5	0
78	4197.12	11.1	FN	5	4
79	4242.12	11.3	FE	2	2
80	4243.12	9.2	FE	1	0
81	4244.12	13.6	ME	4.5	0
82	4317.12	11.8	FE	3	1
83	4316.12	11.7	FE	4.5	0
84	4318.12	11.7	FE	3	0
85	4372.12	13.6	FN	8.5	3
86	4373.12	14.5	FN	6	0
87	4483.12	9	FN	2.5	0
88	192.13	14	MN	8	4
89	2015.13	13	MN	9.5	2

7.2 Appendix 2: CKCS Platelet count and large platelets

Dog	Case number	Platelet count (Advia) ($x10^9$/L)	Platelet count (Manual) ($x10^9$/L)	Large platelet count (Advia) ($x10^9$/L)
1	3294.11	401	486	11
2	482.12	306	295.5	8
3	489.12	28	45	15
4	580.12	129	112.5	30
5	601.12	182	177	34
6	600.12	33	54	20
7	599.12	496	468	10
8	709.12	198	213	4
9	712.12	27	34.5	19
10	716.12	308	289.5	7
11	909.12	332	349.5	2
12	911.12	326	283.5	4
13	912.12	194	180	27
14	913.12	281	280.5	9
15	924.12	277	294	16
16	994.12	184	187.5	18
17	996.12	180	217.5	45
18	995.12	68	51	36
19	999.12	292	256.5	13
20	1000.12	351	315	5
21	1003.12	59	82.5	33
22	1001.12	475	315	5
23	1047.12	535	448.5	5
24	1093.12	332	336	6
25	1100.12	27	37.5	16
26	1101.12	148	174	32
27	1099.12	268	301.5	11
28	1134.12	369	433.5	20
29	1132.12	223	183	24
30	1137.12	254	246	5
31	1138.12	207	240	39
32	1163.12	213	232.5	34
33	1162.12	347	243	9
34	1167.12	163	153	37
35	1165.12	58	55.5	42
36	1164.12	43	34.5	26
37	1166.12	210	262.5	42
38	1190.12	321	336	9
39	1193.12	263	243	6
40	1194.12	239	217.5	20
41	1215.12	378	376.5	7
42	1211.12	272	279	16
43	1220.12	220	195	25
44	1223.12	173	192	15
45	1222.12	176	195	34
46	1228.12	33	49.5	22
47	1274.12	365	339	6

Dog	Case number	Platelet count (Advia) ($x10^9$/L)	Platelet count (Manual) ($x10^9$/L)	Large platelet count (Advia) ($x10^9$/L)
48	1273.12	212	201	15.57
49	1394.12	209	165	7.18
50	1479.12	103	124.5	39.81
51	1478.12	174	204	23.56
52	1476.12	253	288	9.49
53	1477.12	73	96	63.01
54	1480.12	263	249	11.03
55	1483.12	273	280.5	6.59
56	1546.12	44	75	70.45
57	1583.12	177	165	15.82
58	1576.12	220	237	7.73
59	1590.12	179	231	13.41
60	1591.12	144	211.5	28.47
61	1589.12	285	315	2.46
62	1694.12	261	229.5	4.60
63	1697.12	267	220.5	4.49
64	1797.12	191	190.5	7.85
65	1799.12	111	108	19.82
66	1886.12	243	238.5	11.93
67	1902.12	302	273	6.62
68	1885.12	297	345	1.68
69	2001.12	152	132	15.13
70	1997.12	363	354	1.93
71	2003.12	412	307.5	1.46
72	2292.12	40	52.5	70.00
73	2293.12	93	52.5	22.58
74	2439.12	420	409.5	0.48
75	2433.12	565	642	1.42
76	4194.12	311	310.5	5.14
77	4196.12	174	178.5	15.52
78	4197.12	168	181.5	23.81
79	4242.12	99	78	47.47
80	4243.12	40	33	60.00
81	4244.12	293	241.5	5.12
82	4317.12	101	112.5	27.72
83	4316.12	75	78	38.67
84	4318.12	62	82.5	61.29
85	4372.12	550	465	0.91
86	4373.12	53	73.5	64.15
87	4483.12	347	321	2.59
88	192.13	425	444	3.53
89	2015.13	143	181.5	31.47

7.3 Appendix 3: CKCS Additional platelet indices and haematocrit

Dog	Case number	MPC concentration (g/L)	PCDW (g/L)	MPV (fL)	PDW (%)	MPM (pg)	PMDW (pg)	PCT (%)	HCT (L/L)
1	3294.11	203	43.9	8.2	59.4	1.57	0.73	0.33	0.36
2	482.12	213	52.6	8.3	55.3	1.64	0.65	0.26	0.39
3	489.12	197	58	23.5	60.3	2.93	1.31	0.07	0.42
4	580.12	206	53.9	14.9	71	2.41	1.06	0.19	0.44
5	601.12	213	44.3	13.8	75.6	2.34	1.07	0.25	0.42
6	600.12	213	39.2	26.2	52.6	3.36	0.98	0.09	0.46
7	599.12	184	59.7	8.3	53.3	1.36	0.53	0.41	0.41
8	709.12	199	34.6	8.8	52.8	1.67	0.67	0.17	0.43
9	712.12	219	32.3	28.7	46.3	3.53	0.83	0.08	0.4
10	716.12	215	41.2	8.4	53.1	1.71	0.67	0.26	0.43
11	909.12	217	48.8	7.1	44.5	1.46	0.5	0.24	0.41
12	911.12	192	38.9	7.9	46	1.45	0.58	0.26	0.4
13	912.12	241	37.2	11.6	77.8	2.28	1.02	0.22	0.42
14	913.12	230	36.7	8.7	55.7	1.88	0.74	0.24	0.37
15	924.12	207	31.1	10.6	62.4	2	0.81	0.29	0.39
16	994.12	228	31.2	10.6	72.3	2.11	0.94	0.19	0.42
17	996.12	194	31.8	17.2	66.2	2.64	1.05	0.31	0.43
18	995.12	215	32.3	22.9	57	3.22	0.99	0.16	0.46
19	999.12	211	53.7	9.3	60.1	1.77	0.71	0.27	0.46
20	1000.12	206	38.3	7.9	50.5	1.56	0.59	0.28	0.44
21	1003.12	218	27.4	24.7	52	3.4	0.81	0.15	0.4
22	1001.12	192	46	8.1	47.6	1.46	0.54	0.38	0.41
23	1047.12	200	50.7	7.7	49.1	1.43	0.52	0.41	0.37
24	1093.12	215	44.5	7.8	55.7	1.57	0.62	0.26	0.4
25	1100.12	219	38.4	25.3	55	3.31	1.02	0.07	0.42
26	1101.12	205	37.5	14.8	70.9	2.46	1.08	0.22	0.42
27	1099.12	214	36.6	9.6	60.6	1.91	0.78	0.26	0.45
28	1134.12	194	65.6	9.7	59.7	1.62	0.6	0.36	0.38
29	1132.12	223	41.5	10.7	78.2	2.03	0.96	0.24	0.4
30	1137.12	217	39.5	8.4	52.5	1.72	0.65	0.21	0.4
31	1138.12	211	47.5	13.6	70	2.37	1.05	0.28	0.38
32	1163.12	200	33.2	13.8	72.2	2.29	1.02	0.29	0.43
33	1162.12	196	43.3	9.2	55.8	1.68	0.7	0.32	0.46
34	1167.12	220	30.6	14.8	71.4	2.56	1.02	0.24	0.42
35	1165.12	214	34.5	29.4	44.4	3.56	0.85	0.17	0.41
36	1164.12	217	30.5	25.9	48.8	3.48	0.81	0.11	0.42
37	1166.12	219	29.8	14.1	70.1	2.5	1	0.3	0.38
38	1190.12	184	33.8	9.5	53.4	1.67	0.68	0.3	0.42
39	1193.12	191	37.4	9.1	51.5	1.64	0.68	0.24	0.4
40	1194.12	208	44.6	10.6	71.4	1.94	0.92	0.25	0.4
41	1215.12	205	53	8.2	50.5	1.54	0.58	0.31	0.46
42	1211.12	202	37.8	10.9	61.9	2	0.81	0.3	0.43
43	1220.12	217	41.9	11.5	70.5	2.16	0.97	0.25	0.41
44	1223.12	206	39.7	11.4	65	2.11	0.96	0.2	0.43
45	1222.12	215	33.2	13.9	74.6	2.38	1.06	0.24	0.42
46	1228.12	228	29.5	26.6	48.1	3.54	0.78	0.09	0.4
47	1274.12	209	51.7	8.4	51	1.63	0.64	0.31	0.43

Dog	Case number	MPC concentration (g/L)	PCDW (g/L)	MPV (fL)	PDW (%)	MPM (pg)	PMDW (pg)	PCT (%)	HCT (L/L)
48	1273.12	205	63.7	13	62.4	2.23	0.91	0.28	0.34
49	1394.12	220	40.7	10.6	62.7	2.11	0.87	0.22	0.45
50	1479.12	219	37.9	19.8	62.6	3.02	1.02	0.2	0.39
51	1478.12	205	30.5	16.2	68.2	2.62	1.04	0.28	0.39
52	1476.12	208	34.9	11.2	68	2.08	0.93	0.28	0.38
53	1477.12	214	30.4	26.3	48.5	3.47	0.82	0.19	0.37
54	1480.12	204	37.4	11.5	74.2	2.04	0.99	0.3	0.39
55	1483.12	211	41.9	10.3	62.9	1.99	0.89	0.28	0.4
56	1546.12	220	34.7	28.7	44.3	3.59	0.76	0.13	0.4
57	1583.12	219	33.8	12.4	76.1	2.23	1.03	0.22	0.4
58	1576.12	208	46.2	10.8	60.4	2.01	0.84	0.24	0.38
59	1590.12	212	41.1	12.4	68	2.27	0.97	0.22	0.42
60	1591.12	221	33.9	16	73	2.62	1.1	0.23	0.37
61	1589.12	214	44.1	7.7	56.5	1.54	0.65	0.22	0.35
62	1694.12	218	35	9.2	63.1	1.85	0.78	0.24	0.4
63	1697.12	207	36	9.1	63.2	1.76	0.82	0.24	0.39
64	1797.12	212	32.7	10.6	66.5	2.03	0.91	0.2	0.4
65	1799.12	225	34.1	13.4	75.6	2.4	1.07	0.15	0.39
66	1886.12	194	35	12.6	71.8	2.1	0.97	0.31	0.41
67	1902.12	198	36.8	10.4	66.3	1.89	0.91	0.31	0.47
68	1885.12	210	38.3	8.8	45.6	1.75	0.62	0.26	0.46
69	2001.12	214	36.9	12.4	78.1	2.18	1.06	0.19	0.4
70	1997.12	219	38.2	7.3	56.8	1.52	0.64	0.27	0.42
71	2003.12	204	48.1	7.3	54.9	1.4	0.6	0.3	0.42
72	2292.12	217	30.9	27.8	44.7	3.57	0.78	0.11	0.44
73	2293.12	185	52.6	15.8	68.8	2.36	1.18	0.15	0.43
74	2439.12	199	49.3	7.3	52.3	1.48	0.52	0.31	0.37
75	2433.12	198	34.1	8.6	48.4	1.63	0.61	0.48	0.37
76	4194.12	201	68.8	9.4	57.6	1.65	0.6	0.29	0.4
77	4196.12	217	38.2	12.9	69	2.36	0.99	0.22	0.44
78	4197.12	219	41.2	15.1	69.9	2.57	1.05	0.25	0.47
79	4242.12	212	43.8	21.9	57.6	3.13	0.99	0.22	0.41
80	4243.12	222	44.7	25	52.5	3.39	0.96	0.1	0.42
81	4244.12	198	52.4	10.3	57.5	1.84	0.77	0.3	0.39
82	4317.12	190	28	18.5	63.3	2.74	1.03	0.19	0.42
83	4316.12	215	36.4	19.2	62.1	2.98	1.07	0.14	0.43
84	4318.12	221	35.6	25.7	50.6	3.45	0.88	0.16	0.43
85	4372.12	193	53	7.8	48.3	1.39	0.49	0.43	0.38
86	4373.12	213	35.6	25.5	49.7	3.39	0.96	0.14	0.45
87	4483.12	194	50.9	8	58.6	1.44	0.69	0.28	0.44
88	192.13	193	57.2	9.3	56	1.61	0.62	0.39	0.41
89	2015.13	227	41.8	17	64.6	2.86	1.01	0.24	0.44

7.4 Appendix 4: CKCS Echocardiographic indices

Dog	Case number	Jet size (%)	LA:Ao	LVDD	LVDS	Bwt (kg)	LVDDN	LVDSN
1	3294.11	>50	2.05	3.63	2.59	10.9	1.80	1.22
2	482.12	15 to 50	1.21	3.03	2.03	6.6	1.74	1.12
3	489.12	<15	1.57	2.75	2.02	7.9	1.50	1.05
4	580.12	<15	1.38	2.70	1.72	11.6	1.32	0.79
5	601.12	>50	1.40	3.75	2.41	11.2	1.84	1.13
6	600.12	>50	1.88	3.33	2.20	14.56	1.52	0.94
7	599.12	<15	1.35	2.68	1.73	11.9	1.29	0.79
8	709.12	<15	1.19	2.57	1.57	12.76	1.22	0.71
9	712.12	<15	1.45	2.29	1.39	9.91	1.17	0.67
10	716.12	<15	1.24	2.14	1.50	10.1	1.08	0.72
11	909.12	>50	1.40	3.00	1.92	8.09	1.62	1.00
12	911.12	<15	1.16	2.75	1.48	12.7	1.30	0.66
13	912.12	15 to 50	1.28	3.04	1.67	13.9	1.40	0.73
14	913.12	<15	1.37	2.81	1.77	7.5	1.55	0.94
15	924.12	>50	1.69	4.24	2.66	13.5	1.97	1.17
16	994.12	<15	1.12	2.31	1.61	7.1	1.30	0.87
17	996.12	>50	1.27	3.00	1.59	14.6	1.36	0.68
18	995.12	<15	1.06	2.13	1.32	5.5	1.29	0.77
19	999.12	<15	1.17	3.08	2.04	10.2	1.55	0.98
20	1000.12	<15	1.16	2.94	2.05	12.8	1.39	0.92
21	1003.12	<15	1.17	1.94	0.97	7.5	1.07	0.51
22	1001.12	<15	1.23	2.37	1.38	10.9	1.17	0.65
23	1047.12	>50	1.40	3.35	2.12	8.1	1.81	1.10
24	1093.12	>50	2.08	3.18	1.90	10.5	1.59	0.91
25	1100.12	15 to 50	1.06	2.43	1.44	10.2	1.23	0.69
26	1101.12	<15	1.15	2.73	1.78	11.8	1.32	0.82
27	1099.12	<15	1.14	2.64	1.83	17.5	1.14	0.74
28	1134.12	>50	1.38	2.79	1.67	10.3	1.41	0.80
29	1132.12	<15	1.44	2.34	1.73	9.3	1.21	0.86
30	1137.12	<15	1.29	1.91	1.53	7.1	1.08	0.83
31	1138.12	>50	1.22	3.06	2.17	11.2	1.50	1.02
32	1163.12	15 to 50	1.70	2.55	1.77	11.3	1.25	0.82
33	1162.12	<15	1.40	2.31	1.35	6.3	1.34	0.76
34	1167.12	15 to 50	1.40	2.42	1.69	10.3	1.22	0.81
35	1165.12	<15	1.41	2.40	1.45	8.9	1.26	0.73
36	1164.12	15 to 50	1.47	2.29	1.69	6.1	1.35	0.95
37	1166.12	<15	1.38	2.20	1.27	6.3	1.28	0.71
38	1190.12	>50	1.37	2.62	1.94	8.3	1.41	1.00
39	1193.12	<15	1.31	2.00	1.35	8.2	1.08	0.70
40	1194.12	<15	1.41	2.25	1.41	8.8	1.19	0.71
41	1215.12	<15	1.28	3.12	2.08	11	1.54	0.98
42	1211.12	15 to 50	1.63	2.92	1.85	18.2	1.25	0.74
43	1220.12	<15	1.51	2.55	1.47	7.2	1.43	0.79
44	1223.12	<15	1.47	2.57	1.54	6.5	1.48	0.86
45	1222.12	<15	1.35	2.30	1.79	8.9	1.21	0.90
46	1228.12	<15	1.49	3.02	2.13	10.9	1.50	1.00
47	1274.12	<15	1.33	2.49	1.53	11.6	1.21	0.71

Dog	Case number	Jet size (%)	LA:Ao	LVDD	LVDS	Bwt (kg)	LVDDN	LVDSN
48	1273.12	<15	1.76	2.76	1.88	15.7	1.23	0.79
49	1394.12	<15	1.26	2.21	1.34	11.35	1.08	0.62
50	1479.12	>50	1.44	2.52	1.70	8.2	1.36	0.88
51	1478.12	15 to 50	1.51	2.74	1.79	8.1	1.48	0.92
52	1476.12	>50	1.93	3.86	2.08	8.5	2.06	1.06
53	1477.12	15 to 50	1.60	3.33	2.18	8.8	1.76	1.10
54	1480.12	<15	1.39	2.40	1.63	11.2	1.18	0.76
55	1483.12	15 to 50	1.55	1.63	0.97	6.2	0.95	0.55
56	1546.12	>50	1.38	2.81	2.07	9.4	1.46	1.02
57	1583.12	>50	1.36	2.84	1.65	8.8	1.50	0.83
58	1576.12	<15	1.42	2.34	1.75	5.2	1.44	1.04
59	1590.12	<15	1.28	2.80	2.03	10.7	1.39	0.96
60	1591.12	<15	1.38	2.69	1.84	10.9	1.33	0.87
61	1589.12	<15	1.32	2.52	1.63	7	1.42	0.88
62	1694.12	<15	1.30	2.45	1.43	8.2	1.32	0.74
63	1697.12	<15	1.05	2.53	1.64	10.8	1.26	0.77
64	1797.12	<15	1.33	2.37	1.80	5.89	1.41	1.03
65	1799.12	<15	1.47	2.64	1.74	8.5	1.41	0.89
66	1886.12	>50	1.60	3.41	1.90	10.9	1.69	0.90
67	1902.12	<15	1.55	2.38	1.51	9.3	1.23	0.75
68	1885.12	<15	1.16	2.74	1.69	11.9	1.32	0.77
69	2001.12	>50	1.36	2.63	1.67	7.7	1.45	0.88
70	1997.12	15 to 50	1.30	3.02	2.58	9.8	1.54	1.26
71	2003.12	<15	1.39	2.11	1.48	7.15	1.19	0.80
72	2292.12	<15	1.00	1.95	1.67	9.83	1.00	0.81
73	2293.12	>50	1.62	3.74	1.95	9.9	1.90	0.95
74	2439.12	>50	1.39	2.72	1.60	12.4	1.30	0.73
75	2433.12	>50	1.31	3.09	2.09	7.8	1.69	1.09
76	4194.12	<15	1.56	2.60	1.80	8.7	1.38	0.91
77	4196.12	<15	1.44	2.74	1.47	8.9	1.44	0.74
78	4197.12	>50	1.74	3.78	2.21	11.1	1.86	1.03
79	4242.12	<15	1.17	2.31	1.43	11.3	1.13	0.67
80	4243.12	<15	1.08	2.52	1.84	9.2	1.31	0.91
81	4244.12	15 to 50	1.63	3.03	2.29	13.6	1.41	1.00
82	4317.12	<15	1.03	2.98	1.91	11.8	1.44	0.88
83	4316.12	<15	1.49	2.81	1.75	11.7	1.36	0.81
84	4318.12	<15	1.37	2.80	1.87	11.7	1.36	0.86
85	4372.12	>50	1.93	3.97	2.10	13.6	1.84	0.92
86	4373.12	15 to 50	1.41	2.98	2.01	14.5	1.36	0.87
87	4483.12	<15	1.05	2.49	1.91	9	1.30	0.95
88	192.13	>50	1.97	3.35	1.96	14	1.54	0.85
89	2015.13	15 to 50	1.52	2.91	1.84	13	1.37	0.82

7.5 Appendix 5: Control dog Signalment, platelet indices and haematocrit

Dog	Case number	Breed	Bwt (kg)	Sex	Age (Years)	Dog	Platelet count (Advia) ($x10^9$/L)	Large platelet count (Advia) ($x10^9$/L)	MPC concentration (g/L)	PCDW (g/L)	MPV (fL)	PDW (%)	MPM (pg)	PMDW (pg)	PCT (%)	HCT (L/L)
1	2464.12	Dachshund (miniature)	6.4	MN	10	1	222	20	222	46.9	11.5	64.4	2.23	0.86	0.26	0.49
2	1895.12	Greyhound	34	ME	4.5	2	218	3	212	48.7	7.8	53.2	1.55	0.59	0.17	0.52
3	3070.12	Greyhound	29	FN	6.5	3	123	3	220	51.5	8.7	59.3	1.74	0.68	0.11	0.54
4	2336.12	Maltalier	6	FN	13	4	347	4	211	45.7	7.3	50	1.46	0.55	0.25	0.56
5	838.13	Poodle (toy)	10	MN	10	5	305	17	210	65.9	9.7	67.2	1.74	0.74	0.3	0.51
6	2756.12	Shih Tzu X	7.7	MN	14	6	392	10	214	51.9	8.2	58.1	1.61	0.64	0.32	0.51
7	2364.12	Pugalier	13	MN	3.7	7	394	7	211	49.6	7.6	60.5	1.48	0.6	0.3	0.46
8	1451.12	Poodle (toy)	4.6	FN	4.6	8	699	17	209	50.6	8.3	58.9	1.59	0.61	0.58	0.43
9	150.12	Pekingese	4.3	ME	7	9	424	8	184	36.1	8.9	50.4	1.56	0.62	0.38	0.41
10	734.12	Blue Heeler	27.8	MN	13	10	339	14	211	51.3	9.5	57.6	1.84	0.73	0.32	0.41
11	4239.12	Labrador Retriever	28.5	FN	5	11	282	2	205	43.4	6.8	46.1	1.32	0.49	0.19	0.45
12	930.13	Beagle cross	22.9	MN	5.9	12	228	7	200	57	8.8	57.5	1.59	0.67	0.2	0.6
13	931.13	Beagle cross	6	MN	5.9	13	237	6	184	60.5	8.5	60.1	1.36	0.5	0.2	0.59
14	302.13	Poodle (toy)	5.5	MN	5.5	14	315	40	168	73.8	11.7	63.7	1.58	0.61	0.37	0.47
15	3568.12	Shih Tzu X	10.9	FE	10.9	15	280	18	215	51.1	10.4	64.4	1.98	0.82	0.29	0.48
16	1693.12	Kelpie X	24	MN	24	16	214	4	211	47.6	8	53.9	1.59	0.63	0.17	0.49
17	3810.11	Maltese	4.66	FN	10.3	17	394	5	214	49.4	8	47.2	1.6	0.55	0.31	0.46
18	5596.09	Cocker Spaniel	11.2	FN	11.9	18	304	4	233	41.4	7.5	54.8	1.67	0.66	0.23	0.52
19	3008.12	Maltese X	3.2	FE	11.9	19	673	20	199	48.7	9.1	53.7	1.67	0.65	0.61	0.37
20	132.12	Rottweiler X	29.3	FN	9.8	20	249	2	221	40.4	7.8	44	1.65	0.59	0.19	0.53
21	1487.12	Blue Heeler X	33	FN	7.6	21	379	4	213	39.3	8	46.5	1.63	0.62	0.3	0.53
22	501.13	Tenterfield terrier X	6.1	MN	10.11	22	277	12	196	66.1	9.3	58.2	1.58	0.55	0.26	0.47
23	980.13	Schnauzer (miniature)	11.7	MN	5.7	23	333	10	222	43.6	8.3	57.8	1.71	0.7	0.27	0.53
24	211.13	Jack Russell terrier	8.4	FN	10.1	24	261	7	182	35.2	9.1	58.3	1.56	0.65	0.24	0.55
25	337.12	Kelpie X	21.2	FN	8.5	25	447	12	195	64	8.7	53.4	1.51	0.55	0.39	0.52
26	3136.12	Beagle cross	29.5	FN	13.7	26	304	7	201	59.5	8.2	55.3	1.49	0.61	0.25	0.52
27	1239.13	Staffordshire bull terrier	19.5	MN	13.5	27	502	36	196	75.7	10.1	61.2	1.64	0.61	0.51	0.49
28	1543.12	Greyhound	30.7	MN	2	28	143	4	193	44	8.8	57.4	1.57	0.65	0.13	0.54
29	1370.12	Australian Cattle dog	22.7	ME	15.7	29	524	8	190	58.7	8.2	49.2	1.4	0.48	0.43	0.46
30	2365.12	German Shorthaired Pointer	26.6	FE	12.3	30	421	9	209	54.4	8.5	56.8	1.63	0.65	0.36	0.48
31	3512.12	Golden retriever X	23.2	FN	12.9	31	462	19	214	52.6	9.3	60.9	1.79	0.74	0.43	0.38
32	2709.12	Kelpie X	24.4	MN	15	32	388	15	196	65.3	8.8	62	1.51	0.65	0.34	0.5
33	2691.12	Greyhound	30.2	FN	8.4	33	230	7	206	62.3	8.6	54.3	1.58	0.56	0.2	0.52
34	2315.12	Staffordshire bull terrier	33.7	FN	5	34	255	8	177	64.5	8.8	58	1.34	0.51	0.23	0.58
35	3412.12	American Staffordshire terrier	20.5	MN	0.8	35	409	10	194	57.8	8.4	56.1	1.46	0.57	0.34	0.56
36	1369.13	Jack Russell terrier	7	MN	7.5	36	293	10	204	67.5	8.9	55.6	1.61	0.6	0.26	0.57
37	2813.12	Maltese	2.8	FN	4.6	37	549	4	191	54.9	7.6	47.4	1.33	0.46	0.42	0.54
38	1519.13	Labrador Retriever	35.1	FN	4.7	38	252	10	205	67.2	9.4	58.1	1.68	0.65	0.24	0.56
39	198.12	German Shepherd Dog	33.2	FN	12.3	39	330	5	210	50.7	7.8	53.2	1.52	0.59	0.26	0.52