THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par *l'Université Toulouse III - Paul Sabatier*

Discipline ou spécialité : *Pathologie, Toxicologie, Génétique et Nutrition*

Présentée et soutenue par *Charlotte Misbach* **le Mardi 24 Février 2015**

TITRE:

Determination of reference intervals in small size dogs for variables used in veterinary cardiology

Détermination d'intervalles de référence chez les chiens de petit format pour des variables d'utilité en cardiologie vétérinaire

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ACKNOWLEDGMENTS

Tout d'abord, je souhaiterais remercier mes directeurs de thèse, Monsieur le Professeur Hervé Lefebvre et Madame le Professeur Valérie Chetboul pour la confiance qu'ils m'ont accordée en encadrant ce travail de recherche ainsi que pour leur disponibilité et leur aide précieuse. Ce fut un parcours semé d'embûches mais vous avez toujours été là pour moi. Merci.

Je tiens également à exprimer ma gratitude à mes deux rapporteurs de thèse, Madame le Professeur Kathleen McEntee et Monsieur le Professeur Jean-Claude Desfontis. Merci pour le temps que vous avez consacré à la relecture de ce travail et pour vos conseils avisés.

C'est également avec plaisir que je remercie Monsieur le Professeur Michel Galinier d'avoir accepté de présider mon jury de thèse et Monsieur le Professeur Jack-Yves Deschamps de faire partie de ce jury en tant qu'examinateur. Je leur suis très reconnaissante et honorée du temps qu'ils m'ont accordé.

Merci à Monsieur le Professeur Didier Concordet pour sa collaboration essentielle dans la rédaction des articles associés à cette thèse et sa présence à ma soutenance en tant que membre du jury invité.

Merci à toute l'équipe de l'Unité de Cardiologie d'Alfort, Monsieur le Professeur Jean-Louis Pouchelon, Vassiliki Gouni, Emilie Trehiou-Sechi, Amandine Petit, Cécile Damoiseaux, Vanessa Arqued et les anciennes, Anne-Cécile Hoffmann et Alice Leverrier pour leur aide et leur soutien. Merci également à Adriana Rocha et David Balouka pour leur aide et Sylvie Viallefond pour ses conseils.

Un grand merci à Loïc Desquilbet pour son aide et ses nombreuses explications dans le domaine statistique et bien sûr pour sa patiente.

Merci à Jean-François Gauvry pour son aide.

Merci au Professeur Renaud Tissier pour son soutien sans faille.

Merci à Christine Médaille pour son aide dans la réalisation de mon protocole et pour sa participation à la rédaction d'un des articles de ce travail.

Je remercie également le laboratoire Novartis d'avoir financé mon doctorat. Merci particulièrement à Philippe Gruet, Cindy Speranza-Gastal, Jérôme Blanchet et Patricia Nobour pour leur aide.

Enfin, je remercie de tout mon cœur ma famille, mon père et ma mère chéris, ma petite sœur adorée et mon mari que j'aime pour leur soutien et leur amour. Merci également à mes amis proches qui se reconnaîtront !

Je dédie cette thèse à mon frère Sébastien qui nous a quitté, tu resteras toujours dans nos cœurs.

TABLE OF CONTENTS

SUMMARY IN FRENCH

La dégénérescence valvulaire mitrale (MVD) représente 75 à 80% des cardiopathies dans l'espèce canine. Les chiens de petit format, très populaires en France et en particulier en région urbaine, y sont malheureusement prédisposés. La MVD se caractérise par une dégénérescence et une malposition des feuillets mitraux, ayant pour conséquence une régurgitation mitrale (RM) plus ou moins importante. Une RM grave entraine au long court une dilatation des cavités cardiaques (atriale puis ventriculaire) ainsi qu'une augmentation de la pression dans les capillaires pulmonaires conduisant vers l'insuffisance cardiaque congestive (ICC). Les animaux atteints de MVD peuvent rester asymptomatiques pendant une très longue période, mais l'apparition de symptômes d'ICC tels que toux, dyspnée et intolérance à l'effort ou de complications extra-cardiaques telle que l'insuffisance rénale, peut conduire au décès de l'animal. Les techniques d'imagerie ultrasonore comme l'échocardiographie conventionnelle et le Doppler sont les examens de choix pour diagnostiquer la MVD, évaluer la gravité de la RM et ses conséquences sur la morphologie et la fonction cardiaques. De plus, certaines variables échocardiographiques (diamètre atrial gauche, onde proto-diastolique mitrale par exemple) sont significativement corrélées au stade clinique de la maladie. En effet, ces variables permettent d'obtenir des informations précises sur le pronostic de la maladie à court et long terme, seules ou en combinaison avec des biomarqueurs circulants, comme les peptides natriurétiques ou encore certaines variables biochimiques évaluées en routine, comme la créatinine par exemple. En médecine, toute valeur obtenue à la suite d'un examen complémentaire réalisé chez un sujet malade doit être comparée à des valeurs de référence établies chez des sujets sains. En effet, le

but est de déterminer si la valeur observée par le clinicien chez un patient peut être considérée normale ou non. Ce concept d'intervalle de référence (IR) a été introduit en médecine humaine dans les années 60 et suivi par l'élaboration de recommandations spécifiques concernant l'établissement de ces IR par des experts de l'*International Federation of Clinical Chemistry-Clinical Laboratory and Standard Institute* (IFCC-CLSI). En biologie clinique vétérinaire, quelques études ont établi des IR pour des variables biochimiques et hématologiques en suivant ces recommandations dans différentes races ou catégories de races canines. Il ressort de ces études que des spécificités raciales existent ainsi que des effets de certains facteurs, comme le poids, le sexe et l'âge. Concernant le domaine de la cardiologie canine, l'effet de la race et du poids sur les variables échocardiographiques a été largement démontré. Cependant, les effets du sexe et de l'âge demeurent méconnus et aucun IR établi selon les recommandations IFCC-CLSI n'a été publié à ce jour.

L'hypothèse principale de ce travail a donc été la suivante : la détermination d'IR spécifiques chez les races de chiens de petit format les plus populaires en France et prédisposées à la cardiopathie la plus fréquente en cardiologie vétérinaire (MVD), pourrait être pertinente pour le clinicien. Trois études ont été réalisées :

1) Etude 1 : Evaluation des effets de la race, du poids, de l'âge et du sexe sur l'hématocrite ainsi que 15 variables biochimiques de routine et détermination d'IR pour ces mêmes variables dans une population de chiens adultes sains de petit format, selon les recommandations IFCC-CLSI ;

2) Etude 2 : Evaluation des effets de la race, du poids, de l'âge et du sexe sur le biomarqueur plasmatique *N-terminal pro-brain-type natriuretic peptide* (NT-proBNP) et détermination d'un IR pour ce même biomarqueur dans une population de chiens adultes sains de petit format, selon les recommandations IFCC-CLSI ;

3) Etude 3 : Evaluation des effets du poids, de l'âge et du sexe sur 14 variables écho-Doppler et détermination des IR pour ces mêmes variables dans une population de Cavalier King Charles (CKC) adultes sains, selon les recommandations IFCC-CLSI.

Dans les études 1 et 2, une population incluant 154 chiens de 7 races différentes appartenant à des éleveurs de la région Ile de France (CKC, King Charles, Yorkshire Terrier, Caniche, Bichon, Teckel, Shih-Tzu) a été recrutée de manière prospective. Dans l'étude 3, une population de 134 CKC, présentée à l'Unité de Cardiologie d'Alfort pour un dépistage cardiaque a été sélectionnée. Les prélèvements sanguins ainsi que les examens cardiovasculaires ont été réalisés dans des conditions standardisées et en utilisant des méthodes validées, afin de minimiser les sources de variabilité (pré-analytique et analytique pour la biologie clinique ou liée au manipulateur pour l'échocardiographie) qui auraient pu interférer avec l'interprétation des IR.

La détermination des IR a été faite en suivant les recommandations IFCC-CLSI et l'effet des facteurs comme la race, le poids, l'âge ou le sexe sur les variables a été testé en utilisant un modèle de régression linéaire.

Dans l'étude 1, un effet de la race, du poids, du sexe et de l'âge a été identifié, respectivement pour 7/16, 8/16, 4/16 et 3/16 variables. En revanche, la nécessité d'établir des IR spécifiques en fonction de ces facteurs n'a pas montré d'intérêt clinique pertinent. De plus, il est ressorti de cette étude que les IR fournis par les laboratoires d'analyses vétérinaires ne sont pas spécifiquement adaptés aux chiens de petit format. En effet, 4 à 76% des valeurs observées dans la présente étude étaient respectivement au dessus et en dessous des valeurs de l'IR (supérieures ou inférieures) fournies par le laboratoire.

Dans l'étude 2, un effet du sexe a été identifié sur le NT-proBNP plasmatique. En raison de problèmes analytiques et de la grande variabilité inter-individuelle de ce biomarqueur, non décrite jusqu'ici dans la littérature chez les chiens de petit format, il n'a pas été possible de déterminer un IR pour ce biomarqueur (notamment concernant la limite supérieure de cet IR). Il a donc été conclu que l'interprétation d'un dosage plasmatique du NT-proBNP chez un chien sain de petit format doit être réalisée avec prudence et en complément de l'échocardiographie, qui demeure l'examen de choix dans l'évaluation cardio-vasculaire du chien.

Dans l'étude 3, un effet significatif du poids a été identifié sur les variables échocardiographiques, en particulier l'épaisseur des parois et le diamètre des cavités du ventricule gauche. De plus, en comparaison aux IR déterminés dans des études antérieures concernant les mêmes variables échographiques, mais à partir d'une population incluant des chiens de races très différentes, il a été montré que des IR spécifiques élaborés pour le CKC dans la présente étude sont plus précis. Il a donc été conclu que la race et le poids doivent être pris en compte dans l'établissement d'un IR pour les variables écho-Doppler chez le CKC.

En conclusion, ce travail a permis d'identifier l'intérêt de déterminer des IR spécifiques dans une sous-population canine (dans le présent travail, les races de chiens de petit format) pour certaines variables plasmatiques et échocardiographiques. De plus, l'effet de certains facteurs comme le poids, l'âge et le sexe doivent être pris en compte, mais seulement si un intérêt clinique est identifié.

LIST OF ABBREVIATIONS

ACVIM: American College of Veterinary Internal Medicine

ALT: alanine aminotransferase

ALP: alkaline phosphatase

ANP: atrial natriuretic peptide

Ao: aorta

AST: aspartate aminotransferase

ASVCP: American Society for Veterinary Clinical Pathology

BCS: body condition score

B: Bichon

BNP: brain natriuretic peptide

BUN: blood urea nitrogen

BW: body weight

CKCS: Cavalier King Charles Spaniel

CHF: congestive heart failure

CI: confidence interval

CLSI: Clinical and Laboratory Standards Institute

CV: coefficient of variation

D: Dachshund

DMVD: degenerative mitral valve disease

E: early diastolic mitral wave

FS%: fractional shortening

HGB: hemoglobin

IFCC: International Federation of Clinical Chemistry

ISACHC: International Small Animal Cardiac Health Council

KCS: King Charles Spaniel

LA: left atrium

LVED: left ventricular diameter at end-diastole

LVES: left ventricular diameter at end-systole

MCHC: mean corpuscular hemoglobin concentration

MCV: mean corpuscular volume

MR: mitral regurgitation

MP: Miniature Poodle

NT-proANP: N-terminal pro-atrial-type natriuretic peptide

NT-proBNP: N-terminal pro-brain-type natriuretic peptide

PCV: packed cell volume

PLT: platelet

RAAS: renin-angiotensin-aldosterone system

RBC: red blood cell

RI: reference interval

SD: standard deviation

ST: Shih-Tzu

TR: tricuspid regurgitation

TTE: transthoracic echocardiography

VHS: vertebral heart scale

WBC: white blood cell

YT: Yorkshire Terrier

LIST OF TABLES

according to the International Federation of Clinical Chemistry and the Clinical and Laboratory Standards Institute recommendations in clinical pathology.

Table 9. Hematological values of eight sighthound breeds. *LRI, laboratory reference interval; % ALRI, % of dogs above LRI; % ULRI, % of dogs below LRI; Hob, hemoglobin; Hot, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PLT, platelet count; RBC, red blood cell count; WBC, white blood cell count. From Uhríková et al. Hematological and biochemical variations among eight sighthound breeds. Aust Vet J 2013;91:452-459.*

Table 10. Pairwise comparison between breeds indicating significant differences (X). *AM, Alaskan malamute; SH, Siberian Huskies; ES, English Setters; GR, Golden retrievers. From Sharkey et al., Breed-associated variability in serum biochemical analytes in four large-breed dogs. Vet Clin Pathol 2009;38:375-380.*

Table 11. Studies about radiographic evaluation of heart in healthy dogs. *CKCS, Cavalier King Charles Spaniel; SD, Standard deviation.*

Table 12. Studies about electrocardiography in healthy dogs. *CKCS, Cavalier King Charles Spaniel; SD, Standard deviation.*

Table 13. Studies about cardiac biomarkers in healthy dogs. *CKCS, Cavalier King Charles Spaniel; SD, Standard deviation.*

Table 14. Studies about echocardiography in healthy dogs. *CKCS, Cavalier King Charles Spaniel; SD, Standard deviation; ST, speckle tracking imaging; TDI, tissue Doppler imaging; 2D, bidimensional.*

Table 15. Method of analysis for plasma urea, creatinine, total proteins, albumin and liver enzyme activities and results of the between-run coefficient of variations for control solutions.

Table 16. Method of analysis for plasma glucose, electrolytes, phosphate, cholesterol and triglycerides and results of the between-run coefficient of variations for control solutions.

Table 17. Reference ranges proposed for the conventional and pulsed wave Doppler modes variables assessed in the present study. From *Chetboul et al. Use of quantitative two-dimensional color tissue Doppler imaging for assessment of left ventricular radial and longitudinal myocardial velocities in dogs. Am J Vet Res 2005;66:953-961.*

Table 18. Equation used to predict the reference ranges for the 6 body-weight dependent M-mode variables assessed in the present study (i.e., end-diastolic and end-systolic left ventricular wall thicknesses and internal diameters). *From Gonçalves et al. Linear, logarithmic, and polynomial models of M-mode echocardiographic measurements in dogs. Am J Vet Res 2002;63:994-999.*

Table 19. Reference intervals established from the 154 reference individuals and comparison with the laboratory's reference intervals. *BCG, Gaussian after Box-Cox transformation; CI, confidence interval; LL, lower limit; NG, non-Gaussian; UL, upper limit.*

Table 20. Predicted regression-based reference intervals (lower and upper limits) according to body weight for the six M-mode variables with a significant body weight effect assessed in a population of healthy adult Cavalier King Charles Spaniels (n=134). *LVDd and LVDs, end-diastolic and end-systolic left ventricular diameters, respectively; LVFWd and LVFWs, end-diastolic and end-systolic left ventricular free wall thicknesses, respectively; IVSd and IVSs, end-diastolic and end-systolic interventricular septum thicknesses, respectively.*

Table 21. M-mode echocardiographic reference intervals (2.5th and 97.5th percentiles) from 134 healthy adult Cavalier King Charles Spaniels determined according to the Clinical and Laboratory Standards Institute *(CLSI, 2008)* statistical procedures (A), and predictive reference intervals calculated in the same population using Cornell's formula (*Cornell et al., 2004;* B). *See Table 20 for remainder of key.*

LIST OF FIGURES

Figure 5. Kaplan-Meier survival curves according to both International Small Animal Cardiac Health Council (ISACHC) class and plasma concentration of N-terminal pro-brain-type natriuretic peptide (NT-proBNP) obtained from 46 dogs with symptomatic degenerative mitral valve disease: *ISACHC class 2 with NT-proBNP <1265 pmol/L (n= 13, solid line) and >1265 pmol/L (n=10, dashed line); ISACHC class 3 with NT-proBNP <2700 pmol/L (n= 13, dotted line) and >2700 pmol/L (n= 10, alternate dotted and dashed line). n, number of dog. From Serres et al., Plasma N-terminal pro-B-type natriuretic peptide concentration helps to predict survival in dogs with symptomatic degenerative mitral valve disease regardless of and in combination with the initial clinical status at admission. J Vet Cardiol 2009;11:103-121.*

Figure 6. Relationship between the terms defined in the International Federation of Clinical Chemistry and the Clinical and Laboratory Standards Institute recommendations. *From Geffré et al. Reference values: a review. Vet Clin Pathol 2009;38:290.*

Figure 7. Curves showing different types of distribution: bell shape Gaussian distribution (dotted curve) as well as skewness and kurtosis (continuous curves) with the two upper figures showing asymmetric distributions (A and B, positive and negative skewnesses, respectively) and the two lower figures showing distributions with non-Gaussian peakedness (C and D, positive and negative kurtosis, respectively). *From Solberg. Establishment and use of reference values. In: Burtis CA, Ashwood ER, eds. Tietz Textbook of Clinical Chemistry. 3rd ed. Philadelphia, PA: Saunders;1999:336-356.*

Figure 8. Graphic determination of the central 95% reference interval (2.5th and 97.5th percentiles) for a variable expressed in the "*International System of Units*" (SI) by plotting the cumulative distribution.

Figure 9. Serum creatinine concentration in Greyhound *versus* non-Greyhound dogs. *The horizontal white bar represents the median value. The upper and lower limits of the box represent the 25th and 75th percentiles. The whiskers represent the 10th and 90th percentiles. The rounds represent extreme values. From Feeman et al. Serum creatinine concentrations in retired racing greyhounds. Vet Clin Pathol 2003;32:40-42.*

Figure 10. Box-and-whisker plot depicting the serum troponin I concentrations in healthy Greyhound, non-Greyhound and Boxer dogs. *The horizontal bar represents the median value and the whiskers represent the 2.5% and 97.5 percentiles. From LaVecchio et al. Serum cardiac troponin I concentration in retired racing Greyhounds. J Vet Intern Med 2009;23:87-90.*

Figure 11. Boxplots showing distribution of the natriuretic peptides pro-atrial-type (proANP 31-67, Figure 11A) and N-terminal pro-brain-type (NT-proBNP, Figure 11B) by breed. *The top, bottom, and line through the middle of each box correspond to the 75th percentile (top quartile), the 25th percentile (bottom quartile) and the 50th percentile (median), respectively. The whiskers extend from the bottom 2.5th percentile to the top 97.5th percentile. Outliers are represented by black dots. Box, Boxer; BS, Belgian Shepherd; CKCS, Cavalier King Charles Spaniel; Dach, Dachshund; Dob, Doberman Pinscher; Fin L, Finnish Lapphund. From Sjöstrand et al. Breed differences in natriuretic peptides in healthy dogs. J Vet Intern Med 2014;28:451-457.*

Figure 12. Linear regression plot for plasma N-terminal atrial-type natriuretic peptide (NT-ANP) concentrations in 17 healthy Cavalier King Charles Spaniels. *The regression formula is Y = 184.9 + 25.4x with Y, the NT-ANP concentration and x, the age of the dogs. From Eriksson et al. Effect of age and body weight on neurohumoral variables in healthy Cavalier King Charles spaniels. Am J Vet Res 2001;62:1818-1824.*

Figure 13. Body condition system in dogs based on a 9-point scale. *From Nestlé Purina.*

Figure 14. Right parasternal transaortic short-axis view in bidimensional mode showing positioning of calipers (double arrows) allowing calculation of the left atrium (LA) on aorta (AO) ratio. *HR, heart rate (in beat per minute); PA, pulmonary artery.*

Figure 15. M-mode right parasternal left ventricular short-axis view. *HR, heart rate (in beats per minute); IVS: interventricular septum; LV, left ventricle; LVFW, left ventricular free wall; RV, right ventricle. Photograph: Cardiology Unit of Alfort.*

Figure 16. Pulsed wave Doppler mode showing the maximal systolic aortic velocity, on the left apical long axis 4-chamber view (A) and the maximal systolic pulmonary velocity (B), on the right parasternal transaortic short-axis view. *Photograph: Cardiology Unit of Alfort.*

Figure 17. Material used for systemic arterial blood pressure measurement using the Doppler method. *Source: www.dispomed.com*

Figure 18. Distribution of plasma N-terminal-pro-brain-type natriuretic peptide (NT-proBNP) concentrations among the 154 healthy small size dog population. *The thick horizontal bar represents the median (i.e., 783 pmol/L) and the thin horizontal bars represent the first and third quartiles (i.e., 476 and 1232 pmol/L, respectively).*

Figure 19. Comparison between plasma N-terminal pro-brain-type natriuretic peptide (NT-proBNP) concentrations assessed in the 11 outlier dogs at first sampling and 3 months later.

GENERAL INTRODUCTION

The dog was the first domesticated animal *(Ovodov et al., 2011)* and had been the most widely kept working, hunting, and pet animal in the whole human history. In France, there are more than 7.8 millions of dogs including 50% of purebred dogs. Additionally, no less than 25% of French households own at least one dog *(Santé Vet, 2009).* In the canine population, there are more than 300 different canine breeds from small to giant size, among which small size dogs (i.e., dogs with a body weight (BW) lower than 12 kg) are very popular, especially in urban areas *(Société Centrale Canine, LOF Registration Statistics, 2012; American Kennel Club 2011 Dog Registration Statistics).* Among this small size dog population, 7 breeds **(Figure 1, Table 1)**, i.e., Cavalier King Charles Spaniel (CKCS), King Charles Spaniel (KCS), Bichon (B), Dachshund (D), Miniature Poodle (MP), Shih-Tzu (ST) and Yorkshire Terrier (YT) represent almost 13% of all registrations to *"Le livre des Origines Françaises" (Société Centrale Canine, LOF Registration Statistics, 2012)*.

Figure 1. Photographs showing the 7 most common small size breeds in France. *A, Miniature Poodle; B, Yorkshire Terrier; C, Cavalier King Charles Spaniel; D, King Charles Spaniel; E, Dachshund; F, Bichon Frisé; G, Shih-Tzu.*

Table 1. Origin and standard of the 7 most common small size breeds in France. *Source: Société Centrale Canine.*

Breed	Country of origin	Coat	Colour	Weight (kg)	Height (cm)
Miniature Poodle	Germany	Corded or curly, generally groomed (continental, puppy, English saddle or sporting clips)	Black, white, grey, brown, apricot	-	28-35
Yorkshire Terrier	England	Long-haired (usually groomed)	Black and tan	< 3.2	-
Cavalier King Charles Spaniel	United Kingdom	Long-haired	Blenheim, tricolour, black and tan and ruby	5.4-8.2	30-33
King Charles Spaniel	United Kingdom	Long-haired	Blenheim, tricolour, black and tan and ruby	3.6-6.3	23-28
Dachshund	Germany	Short, long and wire-haired	Single-colored, single-colored with spots (merle), single colored with tan points, two-colored	4.0-9.0	-
Bichon Frisé	France and Belgium	Long-haired and curly	White	3.0-7.0	23-30
Shih-Tzu	China	Long-haired	Various shades of gold, white, brown, and black	4.0-7.25	20-28

Although these dogs are highly appreciated by people, they are unfortunately prone to develop degenerative mitral valve disease (DMVD), which is the most common heart disease in dogs. This acquired valvular disease consists of incomplete apposition of the two mitral valve leaflets resulting in chronic- mild to severe- mitral regurgitation (MR). During the course of DMVD, several neuro-hormonal mechanisms may be activated, in order to maintain adequate cardiac output, blood pressure, and tissue perfusion. As the disease worsens, those mechanisms may be deleterious, leading to fluid retention and therefore to the development of congestive heart failure (CHF) and CHF-related symptoms such as cough, dyspnea and exercise intolerance. Even if most of the small size dogs suffering from DMVD remain asymptomatic for years or even in their lifetime, severe complications may occur, such as CHF and alteration of other functions, such as renal function, leading to death or euthanasia because they no longer respond to medical treatments. The standard transthoracic echocardiography (TTE) is considered as the gold standard to assess mitral valve lesions and severity of MR, as well as its consequences on heart morphology and function. Moreover, TTE can be used in combination with evaluation of several blood markers, also called biomarkers, in order to assess severity and complications of DMVD. Additionally, biomarkers may help clinicians to make a prognosis and evaluate medical therapy efficiency. One of the most common biomarker used in veterinary cardiology is the plasma cardiac biomarker N-terminal pro-brain-type natriuretic peptide (NT-proBNP), a peptide synthesized by atrial and ventricular cardiomyocytes in response to increased filling pressures and myocardial wall stress.

The dog represents a unique animal model because there are more than 300 breeds with large phenotypic variations. The morphological differences between canine breeds can be significant, for example a BW may vary from less than 1 kg (Chihuahua dogs) to more than 100 kg (Mastiff dogs). Therefore, the effect of covariates such as breed and BW on blood and

echocardiographic variables in dogs has been extensively studied in the literature. Additionally, numerous other physiological effects have been pointed out, the most common being age and gender. Other effects include neutering status, exercise and body condition, since overweight and obesity increased dramatically in dogs as it does in humans. Owing to these observations, the major hypothesis of this work was that determination of specific reference interval (RI) in healthy small size dogs regarding the most common blood and echocardiographic variables used in the longitudinal evaluation of DMVD could be relevant.

The concept of RI is critical because it helps clinicians to make medical decisions about a patient's test result. A RI is defined as an interval into which 95% of values of a reference group fall *(Solberg et al., 1987a)*. In other words, 2.5% of the values are under the lower limit of this interval and the remaining 2.5% are above the upper limit of this interval, whatever the distribution of the reference values. In human medicine, experts from the International Federation of Clinical Chemistry (IFCC) and from the Clinical and Laboratory Standards Institute (CLSI) wrote guidelines providing definitions and specific recommendations about the determination of RI in clinical laboratory *(Solberg, 1987a, 1987b, 1988; Solberg and Stamm, 1991; Petit Clerc and Solberg, 1987; Dybkaér 1987; CLSI 2008)*. In veterinary medicine, the American Society for Veterinary Clinical Pathology (ASVCP) advised adherence to the IFCC-CLSI guidelines while including recommendations for the determination of population-based RI in animals *(ASVCP, 2012; Friedrichs et al., 2012)*. However, in veterinary medicine, there is a lack of studies focusing on RI determination with respect to these guidelines, especially in dogs, for which the quality of healthcare improves daily while remaining similar to human healthcare.

For this purpose, this work includes three studies, whose main objective was to determine RI according to the IFCC-CLSI guidelines in healthy small size dogs regarding plasma

biochemistry, packed cell volume (PCV), plasma NT-proBNP and standard echocardiographic and conventional Doppler variables.

The present manuscript is divided into chapters as follows:

- Chapter I presents the epidemiological, clinical, pathophysiological and echocardiographic features of canine DMVD, as well as echocardiographic and blood variables commonly used in the assessment of disease severity and prognosis.
- Chapter II describes the recommendations for RI determination in clinical pathology according to the IFCC-CLSI and the ASVCP guidelines.
- Chapter III summarizes the RI previously determined in studies regarding veterinary clinical pathology and cardiology. Additionally, the effect of covariates such as breed, BW, age and gender on clinical pathology and cardiology variables in dogs will also be addressed.
- Chapter IV presents the scientific aim of this work.
- Chapter V describes the material and methods used in the present work to carry the studies.
- Chapter VI summarizes the studies and provides the corresponding original articles.
- Chapter VII is dedicated to the discussion, perspectives and conclusion of this work.

CHAPTER I. THE DEGENERATIVE MITRAL VALVE DISEASE

I. INTRODUCTION

The DMVD, also previously called myxomatous mitral valve disease or mitral endocardiosis, is the most common acquired heart disease in dogs *(Buchanan, 1977; Thrusfield et al., 1985; Pedersen et al., 2000; Häggström et al., 2004; Kvart et al., 2005).* Degenerative lesions of the mitral valve leaflets and associated *chordae tendineae* lead to insufficient coaptation, and therefore a chronic regurgitation through the mitral valve. At physical examination, DMVD is characterized by a left apical systolic heart murmur of varying intensity, i.e., from grade 1/6 to 6/6 according to the *Freeman and Levine's* scale *(1933)*. Progression of the disease is generally slow, especially in small size dogs, and the pre-clinical phase (i.e., the asymptomatic period) may last for years and even for life. Nevertheless, progression is sometimes unpredictable and severe complications may occur, leading to the occurrence of clinical signs related to CHF, and ultimately death or euthanasia because of worsening or unresponsive clinical signs *(Ettinger et al., 1998; Pouchelon et al., 1999; Kvart et al., 2002; Atkins et al., 2007; Borgarelli et al., 2008; Häggström et al., 2008).* Medical treatment of naturally occurring DMVD includes numerous drugs or therapeutic classes, the most common being angiogensin-converting enzyme inhibitors *(Pouchelon et al., 1999; Kvart et al., 2002; Amberger et al., 2004; Atkins et al., 2007; Besche et al., 2007, Pouchelon et al., 2008)*, spironolactone *(Bernay et al.,*

2010; Guyonnet et al., 2010), pimobendan (*Smith et al., 2005; Lombard et al., 2006; Häggström et al., 2008)* and loop diuretics such as furosemide *(Sisson and Kittleson, 1999; De Madron et al., 2011).* More recently, surgical mitral valve repair including suture annuloplasty and *chordae tendineae* replacement has been advocated *(Mizuno et al., 2013).*

In 2009, the American College of Veterinary Internal Medicine (ACVIM) consensus panel formulated guidelines for the diagnosis, classification **(Table 2)** and treatment of the DMVD *(Atkins et al., 2009).*

Table 2. American College of Veterinary Internal Medicine Consensus Statement for classification of dogs with degenerative mitral valve disease. *Atkins et al. Guidelines for the diagnosis and treatment of canine chronic valvular heart disease. J Vet Intern Med 2009;23:1142-1150.*

STAGE A		Patients at high risk for developing DMVD but that currently have no identifiable structural disorder of the heart (e.g., every Cavalier King Charles Spaniel without a heart murmur).
STAGE B	**B1**	Asymptomatic patients with DMVD having no radiographic or echocardiographic evidence of cardiac remodeling in response to DMVD.
	B2	Asymptomatic patients with DMVD having radiographic or echocardiographic evidence of left-sided heart enlargement.
STAGE C		Symptomatic patients with DMVD having past or current clinical signs of heart failure associated with structural heart disease.
STAGE D		Symptomatic patients with end-stage DMVD with clinical signs of heart failure that are refractory to standard therapy.

II. PREVALENCE AND EPIDEMIOLOGY

Prevalence of DMVD is quite high in small size dogs and may attain 14% to 40% depending on the breed and even reach higher values in geriatric canine populations *(International Small Animal Cardiac Health Council 1999; Chetboul et al., 2004a; Serfass et al., 2006).* Both prevalence and progression are believed to be higher and faster in males than females *(Beardow and Buchanan, 1993).* The most common small size breeds known to be predisposed to DMVD include: CKCS, KCS, D, B, YT, ST, MP and Lhassa Apso *(Thrusfield et al., 1985; Pedersen et al., 1999a; Ettinger et al., 1998; Pouchelon et al., 1999; Pedersen et al., 2000; Kvart et al., 2002; Chetboul et al., 2004a; Serfass et al., 2006; Atkins et al., 2007; Häggström et al., 2008; Borgarelli et al., 2008).* Within this small size dog population, the CKCS show specific features, as they develop DMVD at a relatively young age with a high prevalence as compared to other small breed dogs. Nevertheless, the time course of their disease progression to CHF does not appear to be markedly different from that of other small size dogs except for the early age of onset (*Häggström et al., 1992; Beardow and Buchanan, 1993; Chetboul et al., 2004a).* Less commonly, large breed dogs such as German Shepherds may also be affected by the disease and DMVD progression seems to be more rapid in this breed than that observed in small size dogs *(Borgarelli et al., 2004 & 2007).*

III. ETIOLOGY

Little is known about the signaling mechanisms that initiate the pathological process of DMVD in dogs. Such initiating mechanisms may include genetic, chemical and mechanical stimuli *(Buchanan, 1977; Häggström et al., 2004; Madsen et al., 2011),* among which the circulating serotonin concept has recently been studied with interest in dogs with naturally

occurring DMVD *(Gustafsson et al., 2008; Arndt et al., 2009; Oyama et al., 2010; Ljungvall et al., 2013; Cremer et al., 2014).* Other examples of potential mechanisms include heritable connective tissue disorders *(Boudoulas, 2003),* transforming growth factor beta *(Disatian et al., 2009; Aupperle et al., 2008; Obayashi et al., 2011)*, nitric oxyde *(Moesgaard et al., 2007a)* and angiotensin II *(Mow et al., 1999).*

IV. PATHOPHYSIOLOGY

The normal mitral valve apparatus **(Figure 2)** is composed of two leaflets (anterior or septal and posterior or mural), which are thin and transparent *(Buchanan, 1977; Fox, 2012).* Macroscopically, DMVD consist on degeneration of valve leaflets, mainly the anterior *(Tamura et al., 1995),* and associated *chordae tendineae (Trautwein et al., 1973; Liu et al., 1975; Kogure et al., 1980; Terzo et al., 2009)*. The degeneration is characterized by nodular thickening, incomplete apposition of the valve leaflets during systole and secondary MR *(Kittleson et al., 2003; Muzzi et al., 2003; Gouni et al., 2007).* Microscopically, canine DMVD is characterized by increased cellularity in the different leaflet layers, valve structure disorganization, as well as differentiation of valve endothelial and interstitial cells, and glycosaminoglycan infiltration *(Schneider et al., 1973; Buchanan, 1977; Rabkin et al., 2001; Corcoran, et al., 2004; Black et al., 2005; Disatian et al., 2008; Aupperle et al., 2009).*

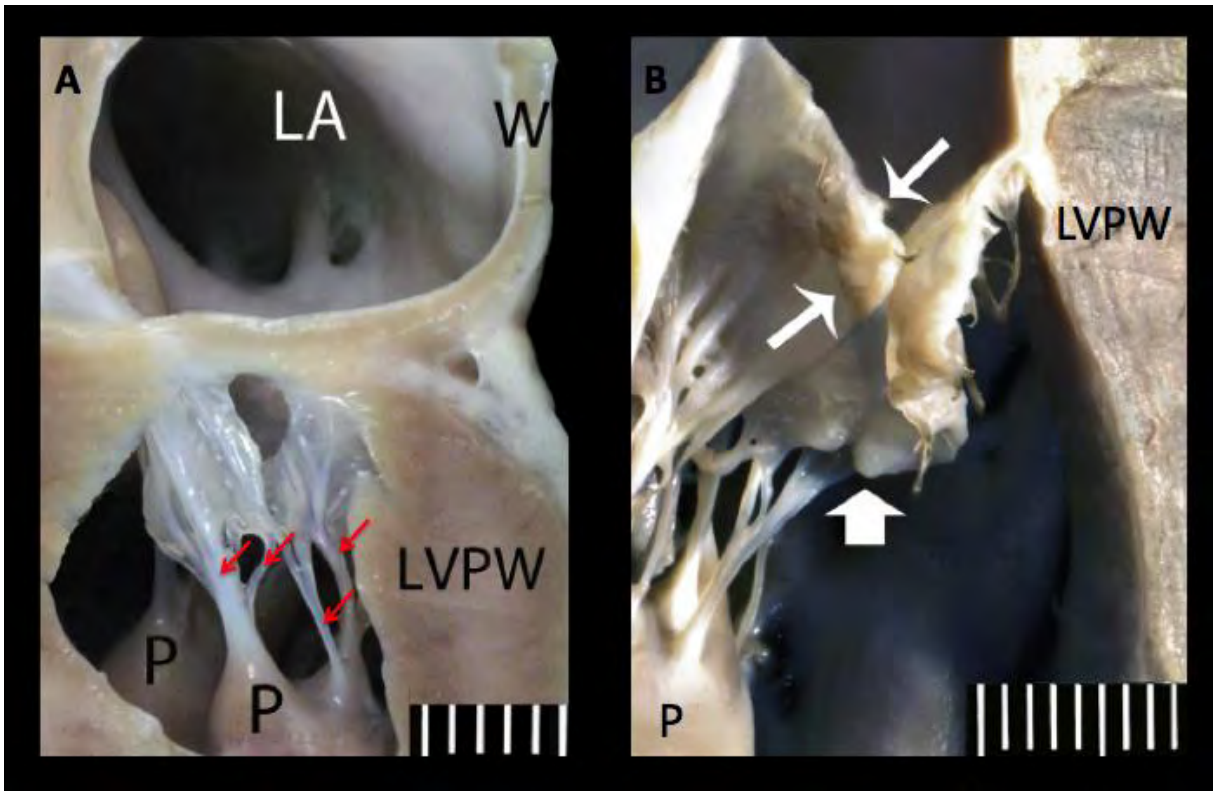

Figure 2. (A) Sagittal section through the left atrium (LA) and the left ventricle of a dog with a normal mitral valve apparatus. *The two mitral leaflets are attached at the junction of atrial and ventricular myocardium. The leaflets are thin, clear and translucent and the chordae tendineae are smooth and symmetric (arrows).* **(B) Close up view of a degenerative mitral valve.** *The leaflet edges are rounded and thickened at their contact point (thin arrows) and nodular thickening (broad arrow) is present along the distal leaflet segments. LVPW, left ventricular posterior wall; P, papillary muscle; W, LA posterior wall. Scale in mm. Photograph and legend: Fox PR. Pathology of myxomatous mitral valve disease in the dog. J Vet Cardiol 2012;14:103-126.*

Depending on MR severity, several potential hemodynamic consequences such as reduced forward cardiac output and increased intracardiac pressures may occur *(Buchanan, 1977; International Small Animal Cardiac Health Council, 1999; Borgarelli et al., 2008)*. In human beings, such hemodynamic alterations may result in complex neurohormonal activation, especially adrenergic nervous and renin-angiotensin-aldosterone system (RAAS) activation, in order to maintain adequate cardiac output, blood pressure, and tissue perfusion *(Francis et*

al., 1990; Ferrari et al., 1998). The prolonged and excessive RAAS and sympathetic activations have deleterious effects with adverse consequences at both cardiac and vascular levels (water and sodium retention, increased heart rate and blood pressure, myocardial and endothelial fibrosis, etc.), which contribute, *inter alias*, to CHF aggravation *(Francis, 1998)* and to occurrence of the cardiorenal syndrome. The cardiorenal syndrome (particularly type II) defines the decline of renal function in the setting of advanced CHF *(Bongartz et al., 2005; Ronco, 2012).*

In dogs with DMVD, few studies have reported the activation of adrenergic system and RAAS, but results remain conflicting *(Ware et al., 1990; Pedersen et al., 1995a; Fujii et al., Häggström et al., 1996 & 1997).* Regarding the occurrence of type-II cardiorenal syndrome in dogs with naturally occurring DMVD, a study showed that the prevalence of azotemia was high (i.e., up to 50%), and increased with CHF severity *(Nicolle et al., 2007)*. Moreover, the glomerular filtration rate decreased by 45% in advanced classes of CHF as compared with the early stages based on the New York Heart Association classification *(Nicolle et al., 2007)*. More recently, another study reported a prevalence of azotemia of 29% and indicated that interlobar renal resistive index increased with CHF severity in 55 dogs suffering from DMVD *(Chetboul et al., 2012a).*

In fine, chronic and severe MR causes enlargement of the cardiac chambers and leads to the development of CHF-related symptoms including exercise intolerance, cough, and dyspnea caused by left-sided CHF *(Häggström et al., 2004; Borgarelli et al., 2008; Atkins et al., 2009),* and ascitis or pleural effusion as signs of right-sided CHF secondary to pulmonary arterial hypertension *(Serres et al., 2006; Stepien, 2009; Chiavegato et al., 2009).*

V. DIAGNOSIS

Although DMVD is characterized at cardiac auscultation by a left apical systolic heart murmur, whose grade is known to be well correlated with MR severity *(Häggström et al., 1995; Pedersen et al., 1999b),* the standard TTE (including bidimensional, M-mode, as well as color, pulsed and continuous wave Doppler) is currently considered as the non-invasive diagnostic method of choice for early detection of mitral valve lesions **(Figure 3)**, evaluation of MR severity **(Figure 4A)** and complications **(Figure 4B)**, and also for assessing its impact on cardiac remodeling, myocardial function, left ventricular filling pressures as well as pulmonary arterial pressure *(Boon, 1983; Chetboul and Tissier, 2012).* The ACVIM consensus statement recommends accordingly that standard TTE should be performed in every dog diagnosed with a left apical systolic heart murmur *(Atkins et al., 2009).* More recently, advanced echocardiographic techniques such as tissue Doppler imaging, speckle tracking imaging and three-dimensional echocardiography have been developed in veterinary cardiology and applied to dogs with DMVD in order to quantify global and regional myocardial function as well as cardiac chamber volumes *(Ljungvall et al., 2011a; Tidholm et al., 2010; Chetboul and Tissier, 2012).*

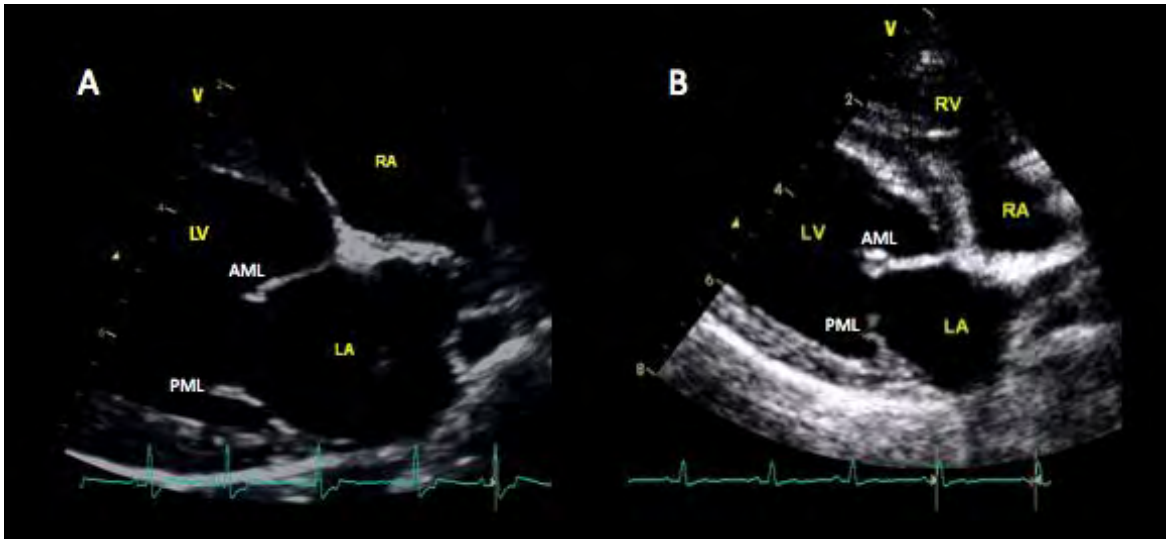

Figure 3. Bidimensional right parasternal long axis 4-chamber view showing echocardiographic images of a normal (A) and a degenerative mitral valve (B). *Note the thickening of the mitral leaflet (B), especially the distal part of the anterior mitral leaflet (AML). LA, left atrium; LV, left ventricle; PML, posterior mitral leaflet; RA, right atrium; RV, right ventricle. Photograph: Cardiology Unit of Alfort.*

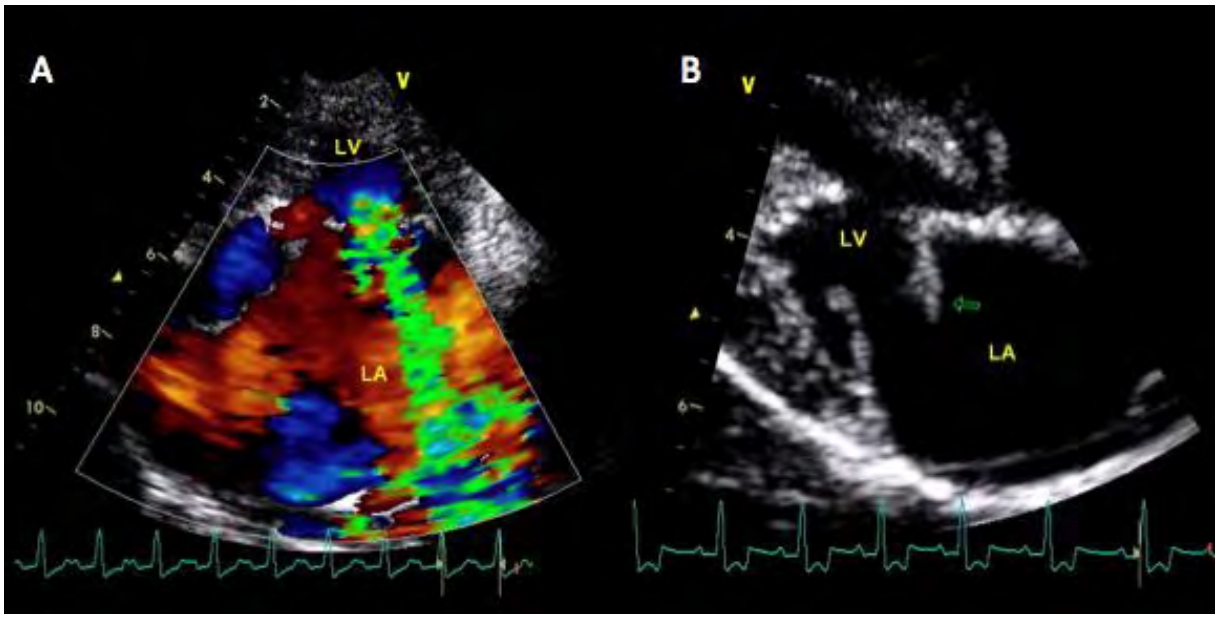

Figure 4. (A) Left parasternal long axis 4-chamber view using the color Doppler mode showing a severe mitral regurgitation. (B) Bidimensional right parasternal long axis 4-chamber view showing a *chordae tendineae* rupture and a prolapse of the mitral anterior leaflet (arrow). *LA, left atrium; LV, left ventricle. Photograph: Cardiology Unit of Alfort.*

VI. PROGNOSTIC FACTORS

VI.1. CLINICAL AND EPIDEMIOLOGICAL FACTORS

Reported clinical and epidemiological risk factors in dogs with DMVD for negative progression of disease and/or death are numerous. A study found that age more than 8 years was associated with a reduced survival time, both when all and only cardiac causes of death

were included *(Borgarelli et al., 2008)*. Moreover, another study showed that survival of class 2 dogs from the International Small Animal Cardiac Health Council *(ISACHC, 1999)* classification (corresponding to stage C of the ACVIM classification) was significantly higher than that of ISACHC class 3 (corresponding to stage D of the ACVIM classification), i.e., 6 months *versus* 157 days *(Serres et al., 2009a)*. A heart murmur grade of 3/6 or greater *(Borgarelli et al., 2008),* as well as being a male (*Beardow and Buchanan, 1993)* is associated with a higher risk of death. Other factors associated with a negative progression of the disease include presence of tachycardia (heart rate >140 bpm), arrhythmias and clinical findings such as syncope, dyspnea, or ascites (*Buchanan, 1977; Egenvall et al., 2006; Serfass et al., 2006; Serres et al., 2007; Borgarelli et al., 2008).*

VI.2. RADIOGRAPHIC AND ECHOCARDIOGRAPHIC FACTORS

Several radiographic and echocardiographic variables are known to be associated with prognosis and outcomes in dogs with DMVD. The first proposed paraclinical marker of potential prognosis interest was heart size, assessed from thoracic radiographs *(Hamlin et al., 1968; Buchanan and Bücheler, 1995)*. Although rapidly replaced by echocardiography, the usefulness of vertebral heart scale (VHS) to predict development of CHF has been reported *(Lord et al., 2011).* There are lots of echocardiographic variables known to affect outcome in canine DMVD. Dogs with a high degree of valve prolapse and severe valve lesions (thickness of the leaflets and *chordae tendineae* rupture) are at higher risk of decompensation and death *(Serres et al., 2007, Terzo et al., 2009).* Moreover, degree of MR, assessed by using the color mapping or the PISA (Proximal Isovelocity Surface Area) methods are useful prognostic factors in asymptomatic and symptomatic dogs with DMVD *(Pedersen et al., 1999a; Olsen et al., 2003; Chetboul et al., 2009).* Variables reflecting the hemodynamic consequences of MR

such as cardiac chamber enlargement, myocardial dysfunction and pulmonary arterial pressure are also commonly used for prediction of CHF and death. Among these variables, heart size (especially left atrium (LA) size, LA on aorta (Ao) ratio and left ventricular internal diameter at end-systole (LVES) and end-diastole (LVED)), fractional shortening (FS%), systolic pulmonary arterial pressure and mitral early (E) wave velocity are the most accurate *(Häggström et al., 1992; Serres et al., 2008; Borgarelli et al., 2008; Moonarmart et al., 2010; Reynolds et al., 2012; Hezzell et al., 2012a).*

VI.3. CIRCULATING BIOMARKERS

During the last decade, the use of circulating biomarkers in combination with routine cardio-vascular examination has been advocated. A biomarker is *"a characteristic (...) objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Atkinson et al., 2001).* There are many biomarkers in veterinary medicine *(Boswood, 2009)* but this paragraph will only focus on biomarkers with a demonstrated clinical usefulness in cardiology.

VI.3.1 NATRIURETIC PEPTIDES

To date, B-type natriuretic peptides, including brain natriuretic peptide (BNP) and its inactive aminoterminal portion of brain natriuretic peptide (NT-proBNP), are considered as being the most reliable neurohormonal markers of heart diseases in dog *(Schober, 2005; Boswood et al., 2008; Oyama, 2009; Sisson, 2009).* Brain natriuretic peptide is secreted as an inactive precursor molecule (proBNP) mainly by atrial and to a greater extent ventricular

cardiomyocytes in response to increased filling pressures and wall stress *(Schober, 2005)*. Inactive proBNP is then cleaved into the circulating biologically active BNP and the inactive NT-proBNP fragment *(Schober, 2005)*. The NT-proBNP has a longer half-life and a better stability in a sample than BNP *(Schober, 2005)*. Therefore, NT-proBNP is the most commonly used natriuretic peptide in veterinary cardiology.

Plasma NT-proBNP concentration is correlated with canine DMVD severity, and may be used in combination with clinical status to predict outcomes in both asymptomatic dogs and dogs with CHF *(Chetboul et al., 2009; Serres et al., 2009a; Tarnow et al., 2009; Takemura et al., 2009; Reynolds et al., 2012; Hezzell et al., 2012b)*. A study *(Serres et al., 2009a)* showed that the median survival time of dogs from ISACHC class 3 with a plasma NT-proBNP concentration >2700 pmol/L was extremely short (5 days), whereas that of dogs from the same class but with NT-proBNP <2700 pmol/L was significantly higher (>6 months, $P<0.0001$, **Figure 5**). Additionally, several other studies showed that plasma NT-proBNP concentration decreased after initiation of medical treatment of CHF, although benefit on survival remains unclear *(Atkinson et al., 2009; Schober et al., 2011; Wolf et al., 2012)*.

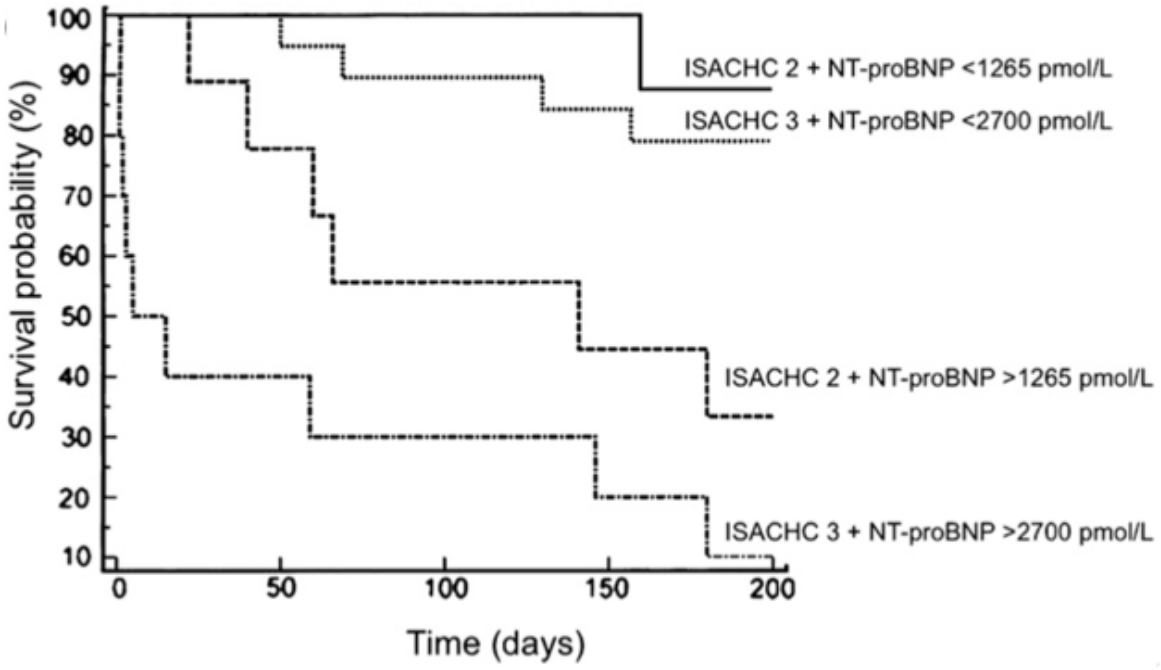

Figure 5. Kaplan-Meier survival curves according to both International Small Animal Cardiac Health Council (ISACHC) class and plasma concentration of N-terminal pro-brain-type natriuretic peptide (NT-proBNP) obtained from 46 dogs with symptomatic degenerative mitral valve disease. *ISACHC class 2 with NT-proBNP <1265 pmol/L (n= 13, solid line) and >1265 pmol/L (n=10, dashed line); ISACHC class 3 with NT-proBNP <2700 pmol/L (n= 13, dotted line) and >2700 pmol/L (n= 10, alternate dotted and dashed line). n, number of dog. From Serres et al., Plasma N-terminal pro-B-type natriuretic peptide concentration helps to predict survival in dogs with symptomatic degenerative mitral valve disease regardless of and in combination with the initial clinical status at admission. J Vet Cardiol 2009;11:103-121.*

Another natriuretic peptide of interest is atrial natriuretic peptide (ANP). Even though this biomarker has been the first identified natriuretic peptide, it has been less extensively studied than NT-proBNP. Several studies demonstrated that plasma ANP concentration is significantly correlated with LA size, severity of disease, and may also help to predict outcomes in dogs with naturally occurring DMVD *(Häggström et al., 1994 & 1997; Greco et al., 2003; Ebisawa et al., 2013).* In a recent report, the median time to CHF in CKCS with N-

terminal-pro-atrial-type natriuretic peptide (NT-proANP) levels >1000 pmol/L was 11 months compared to 54 months in those with concentrations ≤1000 pmol/L *(Eriksson et al., 2014)*.

VI.3.2 CARDIAC TROPONIN I

Cardiac troponin I is a protein exclusively present in cardiac muscle *(Archer, 2003)* and specifically used to detect acute myocardial infarction in humans, as well as marked myocardial damage *(Khan et al., 1999)*. The presence of cardiac disease may lead to myocardial injury and release of troponin I in blood. Serum troponin I concentration is therefore proportional to the severity of myocardial damage *(Boswood et al., 2009)*. In dogs with moderate to severe DMVD, significantly higher concentrations of plasma troponin I were found as compared to healthy controls, and severely affected DMVD dogs had significantly higher concentrations than do mildly and moderately affected dogs *(Ljungvall et al., 2010b; Noszczyk-Nowak, 2011)*. Moreover, a study showed that troponin I was independently associated with survival (all-cause mortality) in dogs with DMVD (*Hezzell et al., 2012b)*.

VI.3.3 CIRCULATING NON-CARDIAC BIOMARKERS

Variables measured as part of standard laboratory investigations, such as hemoglobin (HGB), creatinine, albumin and sodium may help to predict outcomes in human patients with heart disease *(Packer et al., 1987; Cowie et al., 2000; Horwich et al., 2002 & 2008)*. In one study including patients with CHF, the one-year survival was 66% in those with and 83% in those without hypoalbuminemia *(Horwich at al., 2008)*. Similarly, low HGB concentration proved

to be an independent predictor of mortality in human patients presenting advanced stage of heart failure with a low relative risk of 1.113 for each HGB decrease of 1 g/dL *(Horowich et al., 2002).* Concentrations of some of these substances are known to change in response to CHF development and its treatment in canine patients. Hence, dogs with CHF have significant lower, although normal, value of PCV (i.e., 42%) compared with healthy controls (i.e., 45%, *Farabaugh et al., 2004).* Moreover, worsening of CHF is characterized by a significant fall in serum sodium, potassium and chloride (*Boswood and Murphy, 2006)* and a significant increase in the serum urea and creatinine concentrations (*Boswood and Murphy, 2006, Nicolle et al., 2007; Boswood et al., 2009)* in dogs with DMVD. In one previous study *(Boswood and Musphy, 2006),* asymptomatic dogs with DMVD had lower value of creatinine compared to dogs with CHF (respectively, 92 and 127 μmol/L). To the contrary, a fall in serum sodium was found in dogs with advanced CHF compared to healthy dogs (i.e., 140 *versus* 147 mmol/L, respectively). Although these biomarkers are easy to evaluate and known to be correlate with CHF severity, further studies are warranted regarding their prognostic significance.

VII. CONCLUSION

The DMVD is the most common heart disease in small size dogs and has extensively been documented in the veterinary literature since decades. This acquired valvular disease may affect quality and duration of life of dogs to a more or less extend, depending of age of onset, breed, gender, cardiac and extra-cardiac complications and response to medical therapy. Reliable variables measured in routine are known to be correlated with disease severity, help to make a prognosis and monitor efficacy of medical treatment. These variables include echocardiographic variables (e.g., LA size) and blood variables such as creatinine and NT-proBNP.

CHAPTER II. THE CONCEPT OF REFERENCE INTERVAL

I. INTRODUCTION

The concept of RI consists in determining a set of values into which a specific percentage, most often 95%, of the values of a particular analyte in a population would fall *(Solberg et al., 1987a)*. It should be emphasized that RI are often established from general healthy populations. However, sets of values may also be determined from individuals with specific diseases or conditions (children, pregnant women, or smokers for example). These two types of RI are defined as health-related and disease-associated, respectively *(Solberg et al., 1995a et 1995b)*. This RI is then used to help clinicians make the proper medical decisions about their patients' test results. In human beings, the concept of *'normal value'* was introduced by *Grasbeck and Saris* in 1969. Since then, because the term *'normal'* leads to different meanings, i.e., *'normal'* might describe a value common in a population, a value arising from a healthy population or a value which follows a Gaussian distribution in the population *(Grasbeck and Fellman, 1968; Murphy, 1972; Vacha, 1978; Petit Clerc and Solberg, 1987)*, the term *'normal value'* was replaced by the term *'reference value'* to avoid such confusions.

Soon after, the IFCC issued recommendations for the determination of RI that were internationally accepted in the *"Approved Recommendation on the Theory of Reference Values"*

(Solberg, 1987a & 1987b; Petit Clerc and Solberg, 1987; Dybkaér 1987; Solberg, 1988; Solberg and Stamm, 1991). Later, the former recommendations were adopted and updated in 2008 by the CLSI *(CLSI, 2008).* In veterinary medicine, as early as in 1979, several authors applied the concept of RI in animals, such as dogs, horses, cattles and swines *(Lumsden et al., 1979, 1980a & 1980b; Friendship et al., 1984).* The first reported RI established according to the IFCC recommendations was intended for plasma creatine kinase in a population of dogs *(Aktas et al., 1994).* Additionally, the ASVCP recently advised adherence to the IFCC-CLSI guidelines and provided recommendations for the determination of population-based reference RI in veterinary medicine *(ASVCP, 2012; Friedrichs et al., 2012).*

In the *"Approved Recommendation on the Theory of Reference Values",* the IFCC provided the following general definitions (*Solberg, 1987a & 1987b; Petit Clerc and Solberg, 1987; Dybkaér 1987; Solberg, 1988; Solberg and Stamm, 1991)*:

- *Reference individual:* an individual selected for comparison by using defined criteria.
- *Reference value:* the value obtained by measuring an analyte on a reference individual.
- *Observed value:* the measured value for an analyte produced with the aim of making a medical decision by comparison with the reference value.
- *Reference population:* all possible reference individuals.
- *Reference sample group:* adequate number of reference individuals representing the reference population.
- *Reference distribution:* statistical distribution of reference values.
- *Reference limit:* derived from the reference distribution and used for descriptive purposes. Usually, a reference limit is defined so that a stated fraction of the reference values is

inferior than or equal to the limit with a given probability, that is to say the 2.5th or 97.5th percentiles.

- *Reference interval:* interval between and including two reference limits (should not be named *'reference range'* as it represents only one figure).
- *Decision limit:* predetermined threshold that distinguishes 2 populations. Decision limits are defined by consensus and based on investigations on individuals with and without a specific disease or condition.

The relationships between the terms defined above are illustrated in **Figure 6** *(Geffré et al., 2009a).*

After this introduction, the first part of this chapter will focus on the procedures recommended by the IFCC-CLSI (*Solberg, 1987a & 1987b; Petit Clerc and Solberg, 1987; Dybkaér 1987; Solberg, 1988; Solberg and Stamm, 1991; CLSI, 2008)* for determination of population-based *univariate reference values* (i.e., the production, treatment and use of separate reference values for one ore more analyte (s), leading to one or more set (s) of univariate reference values). *Multivariate reference values,* defined as results of two or more analytes obtained from the same reference population that are treated in combination *(Solberg, 1995c; Concordet et al., 2008),* will not be discussed in the present work since this method is not routinely used in veterinary medicine.

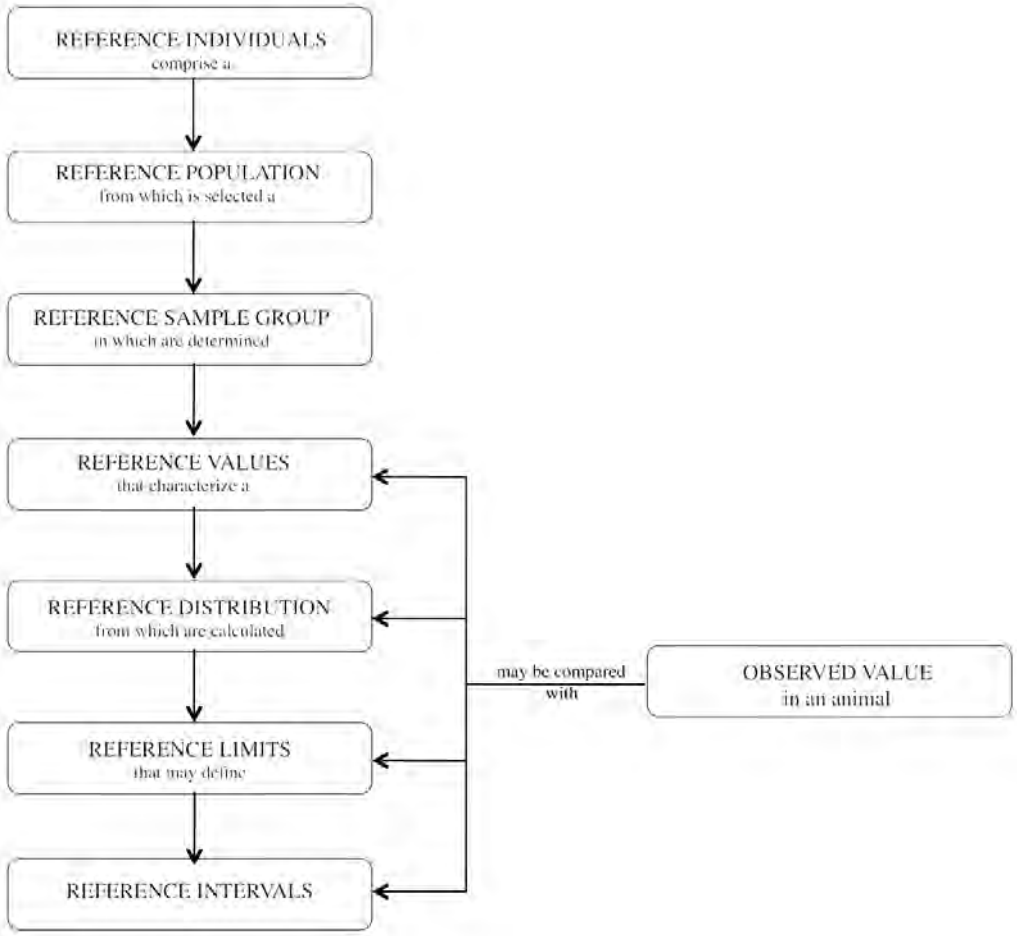

Figure 6. Relationship between the terms defined in the International Federation of Clinical Chemistry and the Clinical and Laboratory Standards Institute recommendations. *From Geffré et al. Reference values: a review. Vet Clin Pathol 2009;38:290.*

II. GENERAL PROCEDURES FOR THE DETERMINATION OF REFERENCE INTERVALS

The IFCC-CLSI (*Solberg, 1987a & 1987b; Petit Clerc and Solberg, 1987; Dybkaér 1987; Solberg, 1988; Solberg and Stamm, 1991; CLSI 2008)* recommendations imply following a step-by-step sequence of operations for RI determination **(Table 3)**.

Table 3. Procedures recommended by the International Federation of Clinical Chemistry and the Clinical and Laboratory Standards Institute for reference interval determination.

1. Establish a list of analytical interferences and source of biological variability
2. Establish selection and partition criteria
3. Execute a written consent
4. Categorize the potential reference individuals (questionnaires)
5. Exclude individuals (exclusion criteria)
6. Decide on appropriate number of reference individuals
7. Prepare the selected subject for specimen collection
8. Collect and handle the biological specimens
9. Collect the reference values by analyzing the specimens
10. Inspect the reference values data distribution and prepare a histogram
11. Identify possible data errors and/or outliers
12. Analyze the reference values
13. Document all the previously mentioned steps and procedures

III. ANALYTICAL INTERFERENCE AND SOURCE OF BIOLOGICAL VARIABILITY

An important preliminary investigation is to define the analyte (s) for which the RI is being determined, as well as the main reason of its measurement *(Solberg, 1999; CLSI, 2008; Jones and Barker, 2008).* Secondly, biological variability should be investigated. Variation may occur at the level of the individual (i.e., intra-individual variability), between individuals (inter-individual variability) and as a result of pre-analytical and analytical imprecision. In dogs, this variability has been studied for biochemical *(Ruaux et al., 2012)*, hematological *(Jensen et al., 1998)* and hemostatic *(Wiinberg et al., 2007)* variables by calculating intra-individual, inter-individual, and analytical coefficients of variation (CV). Results of these studies indicate that high individuality (reflected by the index of individuality, which is the ratio of within-subject to between-subject biologic variability) is present for many routinely measured analytes in dogs. Hence, index of individuality values were between 0.9 and 3.4, 1.03 and 1.40, 0.5 and 0.8 for respectively 14 biochemical, 4 hematological and 6 hemostatic variables, confirming that population-based RI are of significant value in the clinical setting.

By investigating on these potential sources of interference, clinicians can determine specifications regarding:

- The population selection (i.e., inclusion and exclusion criteria);
- The need to separate RI according to criteria (i.e., partitioning);
- The procedure of collection and handling of samples.

IV. SELECTION AND PARTITIONING OF REFERENCE INDIVIDUALS

Firstly, the reference sample group used to determine the RI should be as much as possible representative of the reference population. *Selection criteria* define the desired characteristics of a reference individual whereas *partitioning criteria* are used to further subdivide a reference population into a more refined demographic group *(Petit Clerc and Solberg, 1987).* Setting selection criteria allows determination of which individual should or should not be included in a group of reference individuals. Then, depending on the purpose, the use of partition criteria (e.g., gender, age) may be useful to divide the reference individual group into more homogenous sub-groups *(Petit Clerc and Solberg, 1987).*

In the case of RI determination within a healthy population, the concept of *'health'* should be defined. In human beings, there is no consensus on the definition of health, but *The World Health Organization (Anonymous, 1946)* defines health as *"complete physical, mental and social well-being"*. Nevertheless, this definition hardly applies to human beings and definitely does not apply to animals. Therefore, criteria used to determine the health status must be clearly defined and documented *(Petit Clerc and Solberg, 1987).*

Strategies for the selection of individuals are as follows *(Solberg, 1999; CLSI, 2008):*

- *Direct or indirect*

Direct: individuals are selected from a parent population using specific criteria. Although this *de novo* method is the only one in keeping with the IFCC-CLSI recommendations (*Solberg, 1987a & 1987b; Petit Clerc and Solberg, 1987; Dybkaér 1987; Solberg, 1988; Solberg and Stamm, 1991; CLSI, 2008)*, it is considered as time-consuming and expensive.

Indirect: instead of selecting reference individuals, investigators apply certain statistical methods to analytical values arising from a laboratory database in order to obtain assessments with specific characteristics. For example, determination of RI from large hospital-based data has been developed and represents an interesting way to constitute a reference sample group when direct sample is not possible. Several statistical methods are described *(Bhattacharya, 1967; Solberg, 1994; Ferre-Masferrer et al., 1999; Grossi et al., 2005),* among which the modified Bhattacharya method seems to be the most reliable *(Baadenhuijsen and Smit, 1985; Oosterhuis et al., 1990; Concordet et al., 2009).*

- *A priori or a posteriori (direct methods)*

A priori: individuals are selected for specimen collection and analysis if they fulfill defined inclusion criteria.

A posteriori: method using a database containing both analysis results and information about a large number of individuals. Values are selected if individuals fulfill defined inclusion criteria.

- *Random and non-random*

Random: a process of selection giving each item (individual or test results) an equal chance of being chosen.

Non-random: a process of selection giving each item an unequal chance of being chosen.

- *Partitioning of the reference sample group*

Determination of *partition criteria* in order to divide the reference sample group into more homogenous sub-groups may be requested, but their number should be as low as possible to maintain a sufficient sample size *(Alström et al., 1993).* Procedures used to determine if partitioning is necessary and justified will be discussed later in the manuscript. Examples of the most frequent partition criteria to be used for potential sub-grouping of the reference sample group include age, gender, genetic factors, physiological factors (e.g., reproductive status, pregnancy, lactation) and other factors such as environmental and chronobiological factors *(Alström et al., 1993; Solberg, 1999).*

V. PRE-ANALYTICAL PROCEDURES

Numerous pre-analytical factors may influence generations of reliable RI: preparation of individuals before specimen collection (e.g., acclimatization to the room, room temperature), procedure of specimen collection (e.g., material, position of the patient, training of the investigator) and handling of the specimen before analysis (e.g., centrifugation, temperature,

storage of blood specimens, shipment conditions). Standardization of all pre-analytical procedures is therefore a critical step *(Solberg, 1988).* As an example, a study in dogs *(Collins et al., 2010)* showed that serum NT-proBNP concentration was altered to a greater or lesser extend depending on storage temperature. In this report, serum NT-proBNP concentration was sufficiently increased after 3-months freezing at -20°C (median: 413 *versus* 709 pmol/L, before and after freezing, respectively) and decreased after 24 hours at room temperature (median: 629 after 24 hours at 22.4°C *versus* 1052 pmol/L) to affect clinical interpretation of results *(Collins et al., 2010)*. Although general procedures for control of pre-analytical factors are available in veterinary medicine *(Gunn-Christie et al., 2012),* no specific recommendations are provided according to a given specific analyte. Therefore, the preparation of the subject prior to, and during specimen collection, as well as the procedure itself should be described in sufficient details to allow reproduction by adequately trained staff. This is also strongly recommended in human medicine as soon as a laboratory investigation requires a particular protocol *(Alström et al., 1993; Solberg, 1999).*

VI. ANALYTICAL PROCEDURES AND QUALITY CONTROL

Analytical method should be described in details, including equipment, reagents, calibrators, types of raw data and calculation methods. Estimates of analytical error, i.e., CV and bias, should also be recorded *(Solberg and Stamm, 1991; Alström et al., 1993; Flatland et al., 2010; Gunn-Christie et al., 2012). Analytical error* includes random error (i.e., CV) and systematic error (bias). *Random error*, also called *imprecision*, refers to the variation between repeated

measurements on the same sample. *Systematic error* (also called *inaccuracy*) refers to the difference between the measurement of a quantity and its true value. The true value may be defined by a gold-standard method. All these stages are greatly important for the validation and the transference of a RI (i.e., adoption by a laboratory of previously determined RI).

VII. STATISTICAL TREATMENT OF REFERENCE VALUES

VII.1. INSPECTION OF DISTRIBUTION

Inspection of distribution is an important step in the statistical treatment of reference values because it avoids the misapplication or the misinterpretation of statistical methods. One of the more valid methods for inspection of distribution consists in displaying the reference distribution graphically (histogram prepared manually or using by a computer program). Histogram inspection allows identification of highly deviating values (outliers), shape of the distribution (i.e., Gaussian, skewness and kurtosis, **Figure 7**) and also if bi- or polymodal distributions are present and therefore provides initial information regarding the approximate location of reference limits. Using Boxplots is an interesting method for inspection of distribution but less recommended than histogram *(Solberg, 1987b & 1999).*

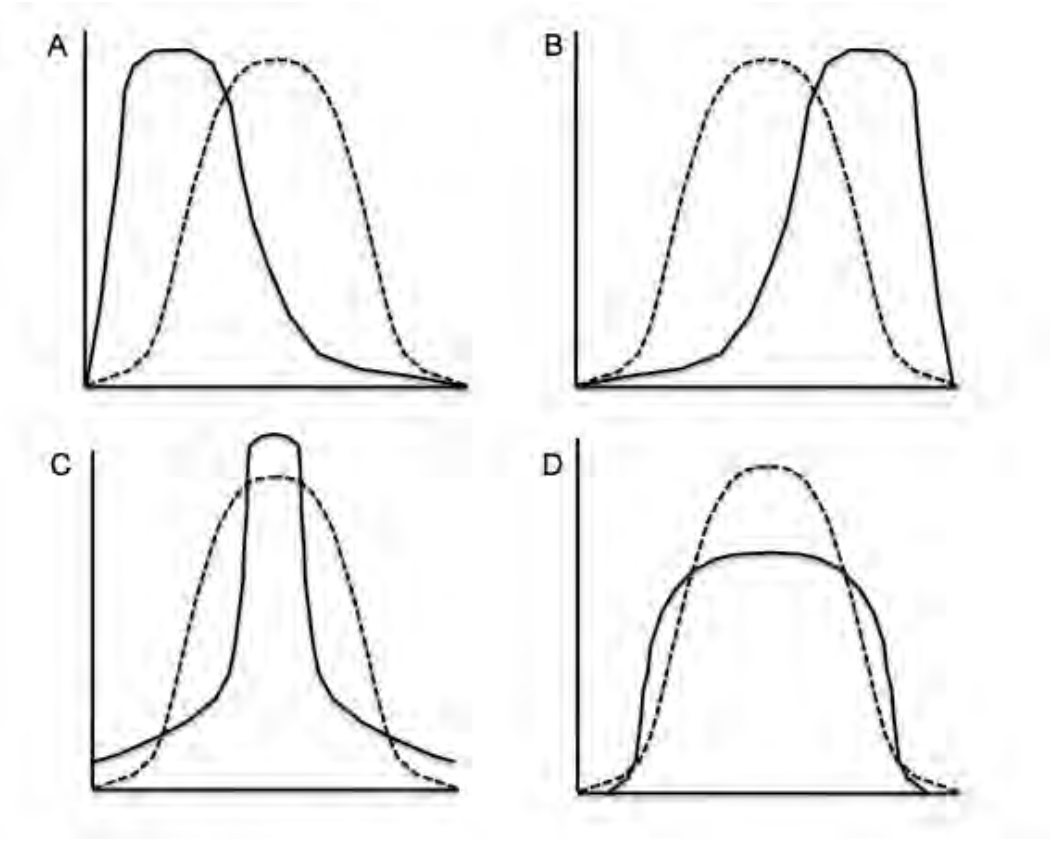

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Figure 7. Curves showing different types of distribution: bell shape Gaussian distribution (dotted curve) as well as skewness and kurtosis (continuous curves) with the two upper figures showing asymmetric distributions (A and B, positive and negative skewnesses, respectively) and the two lower figures showing distributions with non-Gaussian peakedness (C and D, positive and negative kurtosis, respectively). *From Solberg. Establishment and use of reference values. In: Burtis CA, Ashwood ER, eds. Tietz Textbook of Clinical Chemistry. 3rd ed. Philadelphia, PA: Saunders;1999:336-356.*

VII.2. IDENTIFICATION OF OUTLIERS

Outliers are values that do not belong to the underlying distribution of the data. Outliers may result from erroneous inclusion of results from an individual who did not meet the selection

criteria, such as the inclusion of results from a diseased individual. Outliers may also result from pre-analytical, analytical and post-analytical errors. True outliers can affect the location of the reference limits and should be identified and eliminated prior to calculating RI.

The first step in the identification of outliers consists in histogram inspection (see above), followed by the application of statistical methods. Many statistical techniques are available for outlying observations *(Barnett and Lewis, 1978).* Among these tests, the Dixon's outlier range statistic *(Dixon, 1983)* and the Horn's algorithm using Tukey's interquartile fences *(Horn and Pesce, 2003 & 2005)* are the most commonly used.

- The test proposed by Dixon *(Dixon, 1983)* estimates the ratio *R/D*, where *D* is the absolute difference between an extreme observation (large or small) and the next (largest or smallest) observation, and *R* is the range of all observations, including extreme values. If the difference *D* is equal or greater than one-third of the range *R*, the extreme observation is deleted *(Reed and Henry, 1971).*

- The Horn's algorithm using Tukey's interquartile fences identifies multiple outliers located at the upper and lower extremities *(Horn and Pesce 2003 & 2005).* The criterion for rejection is values exceeding interquartile (IQ) fences set at IQ1 - 1.5*IQR and IQ3 + 1.5*IQR (IQR = interquartile range; IQR = IQ3 – IQ1 where IQ1 and IQ3 are the 25th and 75th percentiles, respectively). This test is more stringent than Dixon's range statistic, which favors retention. Once outliers have been eliminated using one or the other statistical test, the remaining data should be retest for additional outliers.

However, an important prerequisite is that data should approximate a Gaussian distribution or be transformed to fit Gaussian distribution *(Horn et al., 2001)* because these procedures may erroneously identify values located in the tail of skewed distributions as outliers. One of the most common methods used for transformation is the method described by Box and Cox *(Box and Cox, 1964; Gillard, 2012).* If data cannot be transformed, the nonparametric approach can be directly applied (see below) for RI determination, as outliers have fewer effects on the RI calculated with this method *(CLSI, 2008).*

Identification of all outliers using the statistical methods described above may not be possible, particularly if several outliers are present in one or both extremities *(Horn et al., 2001; Solberg and Lathi, 2005).* Therefore, a strict selection of the reference sample group as well as a watchful control of pre-analytical and analytical procedures are compulsory in order to minimize the number of "false" outliers. Finally, the decision to include or exclude "true outliers" should be made on a rational basis.

VII.3. SAMPLE SIZE DETERMINATION

Usually, the sample size required for the estimation of the 100α and 100(1- α) percentiles equals to 1/α. Thus, the sample size required for estimation of the 2.5th percentile would be 1/0.025= 40 observations. Nevertheless, as the number of observations increases, precision of percentiles becomes more accurate. A sample size of at least 120 subjects has been advocated *(Solberg, 1987b; Harris and Boyd, 1995; Solberg, 1999; CLSI, 2008)* when using the nonparametric method for determination of RI. For the robust and parametric methods, sample size should be

between 40 and 120 observations. The particular case of very small sample sizes in veterinary medicine will be discussed later.

VII.4. METHODS FOR DETERMINATION OF REFERENCE INTERVAL

VII.4.A. CONDIDENCE INTERVAL FOR REFERENCE LIMITS

The reference limits of a RI are determined in a selected population but are only estimates of the corresponding reference limits in a more general population. Therefore, another sample from the same population would probably yields to different reference limits. A useful way to assess the variability in samples estimates is to associate a confidence interval (CI) to the percentile that is being estimated *(CLSI, 2008)*. In other words, the CI is a range of values including the true percentile (2.5th or 97.5th) with a known probability (usually 90%). The CI are useful because they provide a quantitative measurement of the estimates variability and may narrow as the size of the sampling increases. In the following parts, for each statistical method allowing RI determination, method for calculation of the corresponding CI will be provided.

VII.4.B. NONPARAMETRIC METHOD

Estimation of the 2.5th and 97.5th percentiles using the nonparametric method consists in cutting off a percentage of the values from each tail of the reference distribution. It should be emphasized that Gaussian distribution is not required.

Three techniques have been described:

1. Graphic method: percentiles are determined by plotting the cumulative distribution on a graph paper **(Figure 8)**.

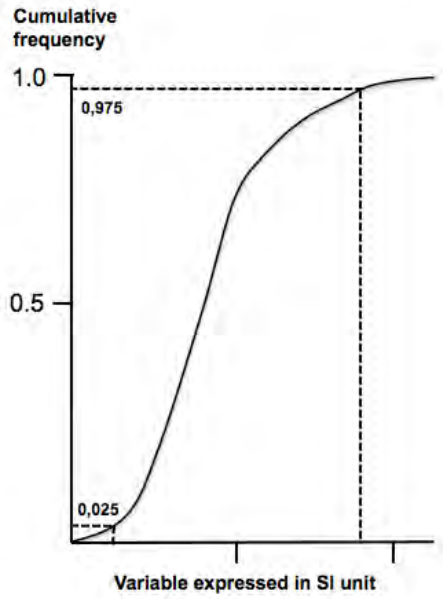

Figure 8. Graphic determination of the central 95% reference interval (2.5th and 97.5th percentiles) for a variable expressed in the "*International System of Units*" (SI) by plotting the cumulative distribution.

2. Mathematic method: a function may be fitted to the reference distribution and percentiles determined by use of the fitted function. In the present work, the nonparametric method was applied for RI determination by using a specific software *(Geffré et al., 2011).*
3. Rank numbers method: ranking numbers turns out to be the simplest and is considered reliable by the IFCC-CLSI (*Solberg, 1987a & 1987b; Petit Clerc and Solberg, 1987; Dybkaér 1987; Solberg, 1988; Solberg and Stamm, 1991; CLSI, 2008)*. To illustrate the nonparametric approach, the procedure using the rank number method will be described according to *Solberg's* description *(Solberg, 1999),* by determining the RI for plasma sodium (expressed in mmol/L) in a 154 healthy small size dog population (see Chapter VI entitled "Results" for details).

The procedure is made up of 5 steps:

a) Sort the n reference values of plasma sodium in ascending order of magnitude (n being the sample size).

b) Rank the values (the minimum value has rank number 1 and the maximum value has rank number n).

In our example, the 2 latter points are summarized in the following charts **(Tables 4 and 5)**. However, to be clearer for the reader, only the 20 first and 20 last out of the 154 observed values of the distribution are presented.

Table 4. Observed values, ranked from 1 to 20, for plasma sodium of the 20 healthy small size dogs present in the left tail of the distribution.

Value (mmol/L)	132	135	136	137	138	138	138	139	140	140
Rank number	1	2	3	4	5	6	7	8	9	10
Value (mmol/L)	140	140	140	140	141	141	141	141	141	141
Rank number	11	12	13	14	15	16	17	18	19	20

Table 5. Observed values, ranked from 1 to 20, for plasma sodium of the 20 healthy small size dogs present in the right tail of the distribution.

Value (mmol/L)	151	151	151	151	151	151	151	151	151	151
Rank number	135	136	137	138	139	140	141	142	143	144
Value (mmol/L)	152	152	152	152	152	152	152	153	154	154
Rank number	145	146	147	148	149	150	151	152	153	154

c) Display the rank number of the 100α and 100(1- α) percentiles as α.(n+1) and (1-α).(n+1), respectively. The limits of the conventional 95% RI have rank numbers equal to 0.025.(n+1) and 0.975.(n+1), with n being the total number of subject in the studied population (i.e., the sample size).

The calculation of rank numbers of the percentiles in our example (i.e., n= 154 healthy small size dogs) is:

Lower: 0.025.(154+1)= 3.87

Upper: 0.975.(154+1)= 151.1

d) Determine the percentiles by finding the original reference values that correspond to the displayed rank, provided the rank numbers are integers. Otherwise, interpolate between the two limiting values.

Here is the original value corresponding to these rank numbers in our example:

Lower reference limit (2.5th percentile): 137 mmol/L

Upper reference limit (97.5th percentile): 152 mmol/L

e) Determine the 90% CI for the 2.5th and 97.5th percentiles using a chart provided by the IFCC-CLSI (Table 6).

In our example, the sample size is 154, therefore:

Regarding the lower reference limit (i.e., the 2.5th percentile), the rank numbers are 1 and 8 **(Table 6)** and the corresponding 90% CI are 132 and 139 mmol/L **(Table 4)**.

Regarding the upper reference limit (i.e., the 97.5th percentile), the rank numbers are:

- (n+1)-upper rank number, i.e., 154+1-8= 147 **(Table 6)**;
- (n+1)-lower rank number, i.e., 154+1-1= 154 **(Table 6)**.

According to **Table 5**, the corresponding 90% CI are 152 and 154 mmol/L.

Table 6. Rank numbers of the 90% confidence interval of the 2.5th percentile for sample size between 119 to 1000 values. *To obtain the corresponding rank number of the 97.5th percentile, subtract the rank numbers in the table from (n+1). n, sample size. From the International Federation of Clinical Chemistry, Expert Panel on Theory of Reference Values: Approved recommendation on the theory of reference values: part 5. Statistical treatment of reference values. J Clin Chem Biochem 1987;25:650.*

Sample size	Rank numbers		Sample size	Rank numbers	
	Lower	Upper		Lower	Upper
119–132	1	7	566–574	8	22
133–160	1	8	575–598	9	22
161–187	1	9	599–624	9	23
188–189	2	9	625–631	10	23
190–218	2	10	632–665	10	24
219–248	2	11	666–674	10	25
249–249	2	12	675–698	11	25
250–279	3	12	699–724	11	26
280–307	3	13	725–732	12	26
308–309	4	13	733–765	12	27
310–340	4	14	766–773	12	28
341–363	4	15	774–799	13	28
364–372	5	15	800–822	13	29
373–403	5	16	823–833	14	29
404–417	5	17	834–867	14	30
418–435	6	17	868–871	14	31
436–468	6	18	872–901	15	31
469–470	6	19	902–919	15	32
471–500	7	19	920–935	16	32
501–522	7	20	936–967	16	33
523–533	8	20	968–970	17	33
534–565	8	21	971–1000	17	34

Summary:

The RI for plasma sodium within a population of 154 small size dogs illustrated by the 2.5th and the 97.5th percentiles and the 90% CI determined using the nonparametric approach are:

Lower reference limit (CI): 137 (132-139) mmol/L
Upper reference limit (CI): 152 (152-154) mmol/L

As illustrated here, this method can be easily performed manually, even if a spreadsheet program may be used.

VII.4.C. BOOTSTRAP METHOD

This method is an extension of the nonparametric method described above *(Shultz et al., 1985; Harris and Boyd, 1995; Linnet, 2000).* Although it is considered as one of the most reliable methods of reference limit and CI determination, a computer is needed because of the heavy re-sampling requested *(Solberg, 1995b).*

The method is as follows *(Solberg, 1999; Henderson, 2005):*

1. Draw, with replacement, random samples of size "n" from the set of "n" reference values. The terms "draw with replacement" mean that each value randomly selected from the set is kept in the set so that it may participate in the random selection of the next value. The number of re-sampling should be high (500 is a reasonable default number).

2. For each re-sample, estimate the upper and lower reference limits (percentiles) by the nonparametric method described above.
3. Compute the mean of the resample estimates of the two reference limits and use the two mean values as the final estimates.
4. The 90% CI of each percentile may be calculated as follows:

$$\bar{x} \pm 1.645 \cdot S_x$$

with $\bar{x}$ and S_x corresponding to the mean and standard deviation (SD), respectively.

In case sample size is inferior to 120 observations, percentiles can be estimated by using the nonparametric approach and the 90% CI by resorting to the Bootstrap method *(CLSI, 2008).*

VII.4.D. PARAMETRIC METHOD

The parametric method is recommended when sample size is comprised between 40 and 120 observations and assumes that the distribution in Gaussian. Moreover, this method usually requires a computer *(Solberg, 1995b)*. The Gaussian distribution of data may be evaluated by using one of the following goodness-of-fit tests *(CLSI, 2008):*

- Anderson-Darling (recommended by the IFCC-CLSI guidelines)
- Kolomogorov-Smirnov
- Shapiro-Wilk
- D'Agostino and Pearson omnibus.

After the normality of data has been tested, there may be three different possibilities:

1. *The distribution is Gaussian*

If distribution has an approximately Gaussian shape, the 2.5th and 97.5th percentiles can be calculated directly and correspond to:

$$\bar{x} \pm 1.960 \, . \, S_x$$

with $\bar{x}$ and S_x corresponding to the mean and SD, respectively.

The 90% CI is estimated as follows:

$$\text{percentile} \pm 2.81 \, . \, S_x/\sqrt{n}$$

with S_x and n corresponding to the SD and number of observations in the reference sample group, respectively.

2. *The distribution is not Gaussian and can be transformed to Gaussian distribution*

If the distribution is not Gaussian, the IFCC-CLSI guidelines recommend transformation by using simple methods (square roots and logarithmic functions) or two-stage methods transformations *(Linnet, 1987),* such as exponential function *(Manly, 1977)* or modulus function *(John and Draper, 1980).* More recently, the Box-Cox transformation has been advocated and recent publications suggested that the generalized Box-Cox family *(Box and Cox, 1964)* could be more advantageous. There are two reasons: first, it achieves a lower bias than the two-stage

transformation; afterwards, the two-stage transformation resulting in percentile estimates may not always be back-transformed to obtain the required RI *(Gillard, 2012).*

After transformation to Gaussian distribution, the estimate of the 2.5th and 97.5th percentiles corresponds to:

$$\bar{y} \pm 1.960 \cdot S_y$$

with $\bar{y}$ and S_y corresponding to the mean and SD of transformed data, respectively.

The final step is reconversion of the percentiles to the original data scale with inverse function transformations and calculation of 90% CI as follows:

$$\text{percentile} \pm 2.81 \cdot S_y/\sqrt{n}$$

with S_y and n corresponding to the SD and number of observations in the reference sample group, respectively.

3. *The distribution is not Gaussian and cannot be transformed to Gaussian distribution*

If the Gaussian distribution cannot be achieved, two alternatives may be used:

a) Estimate percentiles and 90% CI using the nonparametric and bootstrap methods, respectively.

b) Estimate percentiles and 90% CI using the robust approach (see next paragraph), although this method is more powerful when reference data have a symmetrical distribution *(CLSI, 2008; Horn and Pesce 2005).*

VII.4.E. ROBUST METHOD

The robust approach is a compromise between the parametric and the nonparametric methods. It does not require as many observations as the non-parametric procedure (i.e., recommended when sample size is $40 \leq x \leq 120$) and yet does not require that data follow a Gaussian distribution *(Horn, 1999; CLSI 2008).* This method has the same form as the parametric except that it is based on iterative processes estimating the location and spread of the distribution *(Horn and Pesce 1998; Horn et al. 1999).* The initial location (center) is estimated by the median and the initial scale (spread) by the median absolute deviation about the median *(Horn and Pesce, 2005; CLSI 2008).* During the process, observations are down-weighted according to their distance from the central tendency of the sample. In each iteration, the quantity representing the updated estimate of central tendency is calculated until the change in consecutive iterative value is negligible. The advantages of this approach is that firstly, it is more tolerant of outliers in the reference population data and secondly, it does not require a large sample size compared to the nonparametric calculation method. A study showed that the robust statistical analysis was the most reliable method for determination of RI from limited or possibly unreliable data compared to the nonparametric method *(Horn and Pesce, 1998).*

VII.4.F. THE CASE OF SMALL POPULATIONS IN VETERINARY MEDICINE

In veterinary clinical pathology, RI are often determined in small populations such as neonates, special species, zoological species and wildlife animals. A study showed that the results of CI calculations depend on the hypothesis made about the distribution of values, which cannot be

correctly evaluated in small reference sample groups as normality tests lack power. Moreover, when the distribution is skewed, the CI of the upper limit is strongly enlarged as skewness increases, which means that the true limit is located somewhere in a relatively large interval, making users erroneously confident in the upper reference limit *(Braun et al., 2013)*.

Therefore, the *ASVCP Quality Assurance and Laboratory Standards Committee Guidelines for the Determination of Reference Intervals in Veterinary Species* provided specific recommendations regarding determination of RI within small populations *(Friedrichs et al., 2012)*.

1. *Sample size $20 \leq x \leq 40$*

The parametric or robust methods should be used when distribution is Gaussian and non-Gaussian, respectively. Moreover, the following data should be provided to allow clinical decisions to be made *(Geffré et al., 2009b; Friedrichs et al., 2012)*:

- A histogram of the data;
- Mean (in the case of a Gaussian distribution) or median (in the case of a non-Gaussian distribution);
- The minimum and maximum values or a table of all reference values listed in ascending order.

2. *Sample size <20*

Reference interval should not be determined when sample size comprises fewer than 20 observations. However, several data could be disclosed to allow clinical decisions to be made *(Friedrichs et al., 2012)*:

- A histogram of the data;
- Mean (in the case of Gaussian distribution) or median (in the case of non-Gaussian distribution);
- The minimum and maximum values or a table of all reference values listed in ascending order.

VII.5. PARTITIONING

Partitioning reference values into homogenous population sub-groups is an essential part of RI determination as it helps to decrease variability between individuals and narrows the RI *(Lahti et al., 2002a)*. Nevertheless, partitioning should not be carried out if there are fewer than 40 individuals in each sub-group and if the decision to partition is not clinically relevant.

Several methods have been described in the literature:

1. Partitioning is recommended if the absolute difference between sub-group means exceeds 25% of the reference range (range = upper limit – lower limit) of the combined central 95% RI *(Sinton et al., 1986)*. This method requires a Gaussian distribution.

2. Partitioning is recommended if the ratio of sub-group SD (larger SD/smaller SD) exceeds 1.5, regardless of the sub-group means *(Harris and Boyd, 1990)*. If this criterion is exceeded, *Harris and Boyd* recommend that sub-group means should be compared by the standard normal deviate z-test.

Two scenarios are possible:

a) If each sub-group includes at least 120 samples, then partitioning is recommended if $z > 3$ (critical z-statistic). The z value is calculated as follows:

$$z = mean_1 - mean_2 / [(SD_1^2/n_1) + (SD_2^2/n_2)]^{1/2}$$

with SD_1 and SD_2, standard deviation of sub-groups 1 and 2, respectively; n_1 and n_2, number of individuals in sub-groups 1 and 2, respectively.

b) If each sub-group includes less than 120 samples, z should be compared to an alternative critical z-statistic based on the average size (n) of the sub-groups (alternative critical z-statistic). The z value is calculated as follows:

$$z = 3 \times (n/120)^{1/2}$$

This procedure works optimally when the data has a Gaussian distribution and the subclasses are of similar size and SD *(Lathi et al., 2004).*

3. Partitioning is recommended if the ratio of sub-group SD (larger SD/smaller SD) exceeds 1.5. However, if the SD ratio is ≤1.5, the differences between upper (ΔLU) and lower (ΔLL) limits of the 2 sub-groups should take the smaller SD of the subgroups into account *(Lahti et al., 2002b).* Hence, partitioning is recommended if ΔLU, ΔLL or both are ≥ 0.75 of the smaller SD and not recommended if they are < 0.25 of the smaller SD. Again, this method requires Gaussian distribution.

4. Partitioning is recommended if more than 4.1% or less than 0.9% of a sub-group falls outside the upper or lower limits of a combined RI. On the contrary, if the proportion of the sub-group falling outside the combined RI is comprised between 1.8% and 3.2%, partitioning is not advised *(Lahti et al., 2004).* This method has been proposed to partition non-Gaussian reference data into sub-groups.

5. Partitioning may be recommended on the basis of non-statistical criteria. Hence, partitioning should be considered if the literature indicates important clinical differences between sub-groups or if a clinical relevance may be identified *(Harris and Boyd 1990; CLSI, 2008).* To the contrary, partitioning into sub-groups should not be undertaken if the physiological differences would not result in important clinical differences in RI. Therefore, the process of partitioning should be viewed as a serial process including statistical and clinical evidence *(Kjelgaard-Hansen, 2010)*.

VIII. CONCLUSION

In conclusion, a "good" RI allows correct inclusion of most of the subjects with characteristics similar to the reference group and excludes the others *(Ceriotti, 2007).* Factors of variation known to be responsible for this misclassification are intra-individual, inter-individual and analytical *(Schneider, 1960; Harris, 1974).* The statistical method used to determine the RI mainly depends on sample size and distribution of reference values. Therefore, strict procedures should be applied to determine a reliable RI and precise reference limits. Furthermore, presentation of results in a clear and concise laboratory report is strongly advised in order to facilitate transference and validation of RI from other laboratories and also to help clinicians in making the most appropriate medical decisions. Finally, although determination of RI is based on statistical methods, the associated decision limits (i.e., diagnosis thresholds that are used to discriminate between patients with or without disease) should be defined by consensus established from clinical investigations *(Jorgensen et al., 2004; Plebani, 2004).*

CHAPTER III. REFERENCE INTERVALS IN VETERINARY MEDICINE

I. INTRODUCTION

As in human patients, every variable assessed in an animal should be compared with a RI previously established within a reference population, in order to find out if the observed value can be considered normal or not. Behind this simplicity lies the complexity to interpret such a result because biological variables are subjected to high variability, owing to the patient himself (breed, age, BW, height, gender, physical status etc.), but also to pre-analytical and analytical factors. Moreover, in veterinary medicine, there are plenty of diverse species such as mammals, avians, reptiles and there are also lots of phenotypical variations within a specific species. This observation has been already emphasized in dogs with BW varying from less than 1 kg to more than 100 kg, depending on the breed. Therefore, determination of population-based RI is crucial to interpret in the best possible way a veterinary patient test's result. Most of the RI established in veterinary medicine concerns the clinical pathology (mainly hematology and biochemistry) but other specialties are also involved, including diagnostic techniques such as radiology and echography. However, studies on RI determination according to the IFCC-CLSI recommendations in veterinary medicine are lacking, except in veterinary clinical pathology, for which this methodology has been increasingly used since the last two decades.

II. REFERENCE INTERVALS IN VETERINARY CLINICAL PATHOLOGY

II.1. INTRODUCTION

In veterinary clinical pathology, a lot of reports illustrated RI determination in wild and domestic animals using different statistical methods. However, the first reported RI established according to the IFCC recommendations was for plasma creatine kinase in a population of 232 healthy dogs *(Aktas et al., 1994).* Since then, several studies have followed the IFCC-CLSI guidelines for RI determination in healthy populations of dogs, cats **(Table 7)** and other animal species **(Table 8)**. Moreover, determination of RI in a diseased population of dogs according to the latter guidelines has been undertaken, as shown in one previous study including 192 dogs with CHF. In the latter report, RI were established for several serum variables (i.e., urea, creatinine, sodium and potassium) in dogs with DMVD *(Lefebvre et al., 2013).*

Within the canine species, since large phenotypic variations have been observed, the effect of breed on clinical pathology variables has been well-documented and is known to affect interpretation of laboratory test results in a more or less degree. Therefore, it is now consensual that breed-specific RI should be established for some biochemical and hematological variables in dogs. Since the BW is strongly dependent of the breed, this effect may often be confounded by the breed and difficult to identify. Even so, two studies reported the effect of BW on serum/plasma creatinine in dogs (*Médaille et al., 2004; Craig et al., 2006).* Finally, effect of other covariates (e.g., age and gender) has been reported, but the need for partitioning a reference sample group according to the latter covariates remains unclear.

Table 7. Studies about the determination of reference intervals in healthy dogs and cats according to the International Federation of Clinical Chemistry and the Clinical and Laboratory Standards Institute recommendations in clinical pathology.

Authors	Analyte	Breed	Number of animals	Method
Reynolds et al., 2008	Plasma biochemistry	Adult Domestic shorthair cats	95	Parametric and nonparametric
Sharkey et al., 2009	Serum biochemistry	Four adult large-breed dogs	304	Nonparametric
Geffré et al., 2010	Coagulation markers	Adult pure-breed dogs	139	Nonparametric
Nielsen et al., 2010	Plasma biochemistry and hematology	Adult Bernese Mountain dog	32	Robust
Reynolds et al., 2010	Plasma biochemistry	Five adult pure-breed cats	536	Nonparametric
Bourgès-Abella et al., 2011	Hematology	Adult pure-breed dogs	132	Nonparametric
Campora et al., 2011a	Hematology	Adult Greyhound	304	Nonparametric
Campora et al., 2011b	Hematology	Adult Greyhound and Lurcher	217	Nonparametric and robust
Dunlop et al., 2011	Plasma biochemistry	Adult Greyhound	499	Nonparametric
Nikolic Nielsen et al., 2011	Coagulation markers	Adult Bernese Mountain dog	32	Robust method
Sako et al., 2011	Plasma biochemistry	Dogs <1 year old	896	Parametric and nonparametric

Authors	**Analyte**	**Breed**	**Number of animals**	**Method**
Rosset et al., 2012	Plasma biochemistry and hematology	Borzoi and Beagle puppies	31	Parametric and nonparametric
Serra et al., 2012	Hematology	Adult medium and large-breed	259	Nonparametric
Heilmann et al., 2013	Serum alpha-1-proteinase inhibitor	Dogs	87	Nonparametric
Lavoué et al., 2013	Plasma biochemistry	Adult Dogue de Bordeaux	62	Nonparametric
Lavoué et al., 2014	Hematology	Adult Dogue de Bordeaux	58	Nonparametric

Table 8. Studies about the determination of reference intervals in different healthy animal species according to the International Federation of Clinical Chemistry and the Clinical and Laboratory Standards Institute recommendations in clinical pathology.

Authors	Analyte	Species	Number of animals	Method
Knowles et al., 2006	Hematology and biochemistry	Cultured shortnose sturgeon	77	Nonparametric
Thoresen et al., 2009	Hematology and biochemistry	Scandinavian Grey wolve	79	Parametric
Klem et al., 2010	Hematology and biochemistry	Pig	104	Nonparametric and parametric
Cooper-Bailey et al., 2011	Hematology and biochemistry	Gila monster	14 wild and 2 captive	Descriptive statistics
Dawson et al., 2011a	Biochemistry	Alpaca	74	Nonparametric
Dawson et al., 2011b	Hematology and coagulation markers	Alpaca	68	Nonparametric
House et al., 2011	Coagulation markers	Alpaca	29	Descriptive statistics
Hein et al., 2012	Hematology, biochemistry, serum total thyroxine	Ferret	111	Robust
Young et al., 2012	Hematology and biochemistry	Wild Australian tree frog	146	Nonparametric bootstrap
Niedźwiedź et al., 2013	Biochemistry	Polish horse	74	Robust
Matsche et al., 2014	Hematology and biochemistry	Captive Atlantic Sturgeon	119	Robust
Rossi et al., 2014	Hematology and biochemistry	Hedgehog	50	Nonparametric bootstrap

II.2. EFFECT OF COVARIATE ON LABORATORY VARIABLES IN DOGS

II.2.A. BREED EFFECT

As stated in the introduction, the breed effect is the most commonly reported effect on blood variables within the canine species.

1. Hematology

A recent study involving more than 6000 dogs from 75 different breeds demonstrated that all hematological variables, except the mean corpuscular hemoglobin concentration (MCHC), showed significant differences between specific individual breeds and mixed breeds *(Lawrence et al., 2014).* However, this breed effect has been importantly investigated in sighthound dogs and especially in Greyhounds (*Zaldívar-López et al., 2011; Lefebvre, 2011).* Hence, as compared to the general canine population, mean and RI in this special breed category were higher for red blood cell (RBC) count, HGB, PCV and mean corpuscular volume (MCV), whereas they were lower for white blood cell (WBC) and platelet (PLT) counts *(Porter and Canaday 1971; Campora et al., 2011a & 2011b; Uhríková et al., 2013).* Indeed, in a study *(Campora et al., 2011a),* mean HGB concentration was higher in Greyhounds (19.8 g/dL) compared to non-Greyhound dogs (17.1 g/dL) from another report *(Moritz et al., 2004).* In another study, mean WBC and PLT counts were lower in Greyhounds compared to a non-Greyhound group (5.58 *versus* 10.55 x 10^9/ L and 214 *versus* 338 x 10^9/L, for WBC and PLT counts, respectively).

Similar hematologic differences were observed in Galgos Españoles (Spanish Greyhound), although these differences were not as striking as in Greyhounds (*Mesa-Sanchez et al., 2012)*. Interestingly, another study showed that more than 78% of Greyhounds had RI regarding PLT count below those established in a general population of dogs (*Uhríková et al., 2013)*. Additionally, differences within sighthound breeds have also been documented (*Uhríková et al., 2013; Campora et al., 2011b)*, as shown in **Table 9**. The most significant difference was observed for mean PLT count, which was 2 times higher in Azawakh dogs than in Greyhounds (*Uhríková et al., 2013)*. Furthermore, within the sighthound dog category, Lurchers showed higher RI for RBC count (6.74-9.90 x 10^{12}/L) compared to Greyhounds and non-Greyhounds *(*6.67-9.30 and 5.85-8.40 x 10^{12}/L, respectively, *Campora et al., 2011b).*

Table 9. Hematological values of eight sighthound breeds. *LRI, laboratory reference interval; % ALRI, % of dogs above LRI; % ULRI, % of dogs below LRI; Hb, hemoglobin; Hct, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PLT, platelet count; RBC, red blood cell count; WBC, white blood cell count. From Uhríková et al. Hematological and biochemical variations among eight sighthound breeds. Aust Vet J 2013;91:452-459.*

Breed	WBC (×10⁹/L)		RBC (×10¹²/L)		Hb (g/L)		Hct (%)		MCV (fL)		MCH (pg)		MCHC (g/L)		PLT (×10⁹/L)	
	Mean ± SD Range	% ULRI % ALRI	Mean ± SD Range	% ULRI % ALRI	Mean ± SD Range	% ULRI % ALRI	Mean ± SD Range	% ULRI % ALRI	Mean ± SD Range	% ULRI % ALRI	Mean ± SD Range	% ULRI % ALRI	Mean ± SD Range	% ULRI % ALRI	Mean ± SD Range	% ULRI % ALRI
Whippet (n = 47)	5.8 ± 1.5	57.4	7.8 ± 0.8	0	189 ± 18	0	56.1 ± 5.0	0	72.3 ± 3.4	0	24.3 ± 1.5	4.3	337 ± 16	2.1	176 ± 42	73.8
	3.3–10.4	0	5.9–9.9	17	153–228	72.3	44.0–65.6	59.6	65.7–80.1	23.4	21.5–27.4	34	298–364	44.7	82–270	0
Greyhound (n = 27)	5.4 ± 1.8	70.8	7.7 ± 0.5	0	191 ± 15	0	56.2 ± 4.0	0	73.4 ± 1.6	0	24.9 ± 0.8	0	339 ± 12	0	173 ± 49	78.3
	2.7–10.0	0	6.6–8.6	8.3	161–222	70.8	46.8–62.8	58.3	70.3–76.2	20.8	23.6–26.6	45.8	313–359	50	84–285	0
Italian Greyhound (n = 24)	8.1 ± 1.9	8.7	7.6 ± 0.6	0	174 ± 14	0	51.9 ± 4.1	0	68.8 ± 2.2	0	23.1 ± 1.4	0	335 ± 16	0	278 ± 85	17.4
	5.1–12	0	6.4–8.8	4.4	152–198	34.8	44.0–58.0	21.7	65.6–73.8	0	20.0–25.3	4.4	302–361	39.1	129–448	0
Borzoi (n = 23)	11.5 ± 2.3	0	7.7 ± 0.8	0	177 ± 18	0	54.0 ± 5.7	0	70.3 ± 2.4	4.3	23.1 ± 1.2	8.7	327 ± 15	4.3	209 ± 60	35
	6.6–16.5	0	6.0–8.8	13	144–204	43.5	43.6–64.1	43.5	64.6–75.0	0	20.8 + 26.1	4.3	290–347	21.7	72–309	0
Pharaoh Hound (n = 28)	8.8 ± 1.9	11.1	7.5 ± 0.7	0	177 ± 20	0	52.8 ± 4.8	0	70.4 ± 2.8	3.6	23.6 ± 1.8	25	335 ± 20	0	302 ± 153	22.2
	4.8–11.8	0	5.9–9.0	10.7	132–218	42.8	42.2–62.3	32.1	64.0–75.0	0	20.9–27.2	17.8	309–377	42.9	102–726	11.1
Saluki (n = 20)	7.6 ± 1.6	12.5	8.0 ± 0.8	0	185 ± 22	0	55.0 ± 5.7	0	68.9 ± 2.6	6.3	23.2 ± 1.6	18.7	335 ± 15	6.3	211 ± 81	31.3
	4.9–10.2	0	6.1–8.9	37.5	145–211	68.8	44.8–62.3	56.3	63.5–73.4	0	19.6–25.2	6.3	297–355	37.5	84–361	0
Sloughi (n = 13)	9.8 ± 1.7	0	8.1 ± 0.9	0	189 ± 17	0	56.5 ± 4.9	0	70.2 ± 1.9	0	23.5 ± 1.2	7.8	335 ± 15	0	263 ± 92	23.1
	7.1–14.1	0	6.6–9.9	23	165–222	61.5	47.6–64.2	61.5	67.4–73.4	0	21.7–26.2	7.8	306–358	30.7	106–402	0
Azawakh (n = 10)	6.8 ± 1.1	20	8.2 ± 0.9	0	193 ± 16	0	55.6 ± 5.9	0	68.8 ± 2.1	0	23.7 ± 0.9	0	344 ± 7	0	333 ± 107	10
	4.7–8.5	0	6.7–9.4	40	167–217	70	42.4–63.5	60	65.8–71.6	0	22.1–25.0	0	333–355	70	107–500	0
LRI	6.0–17.0		5.5–8.5		120–180		37–55		65–75		22–25		300–340		200–500	

Several other studies pointed out breed particularities in other canine breeds. Firstly, in CKCS, low PLT count (less than 150 x 10^9/L) and greater platelet diameter were observed as compared to other breeds, without evidence of excessive bleeding problems, suggesting physiological macrothrombocytopenia *(Brown et al., 1994).* Similarly, this macrothrombocytopenia was described in a group of healthy Norfolk Terriers from Northern Italy and also in isolated cases in Cairn Terriers *(Gelain et al., 2014).* However, as far as 1999, a leucopenia was documented in 6 healthy Belgian Tervurens *(Greenfield et al., 1999).* Soon after, the same authors *(Greenfield et al., 2000)* performed a study including 180 healthy Belgian Tervurens and showed that, compared to other breeds, Tervurens had lower mean WBC (7.04 *versus* 9.03 x 10^9/L), neutrophil (4.05 *versus* 5.50 x 10^9/L) and monocyte (0.65 *versus* 0.70 x 10^9/L) counts as well as higher mean RBC count (6.62 *versus* 6.45 x 10^{12}/L) and PCV (46.5 *versus* 44.7%).

In another report, 10 routine hematological variables were assessed in 32 Bernese Mountain dogs (*Nielsen et al., 2010)*. In 8 out of the 10 tested variables, standard laboratory RI were considered suitable. However, *de novo* establishment of new RI was undertaken for 2 variables because RI in Bernese dogs were higher than those provided by the standard laboratory (MCHC: 21.2-22.2 *versus* 19.2-21.0 nmol/L; eosinophil count: 0-1.5 *versus* 0-1.2 x 10^9/L, in Bernese and other breeds, respectively). Same observations were found in a population of 96 North American Scottish Deerhounds *(Sheerer et al., 2013)* with a majority of hematological results similar to the generic intervals except for PLT count, for which more than 50% of observed values were below the nonbreed-specific RI. One more study pointed out an isolated hematological difference in 3 large breeds compared to the general population (*Bourgès-Abella et al., 2011)*. Hence, median eosinophil counts were higher in Brittany Spaniels (1.87 x 10^9/L), Rottweilers (1.41 x 10^9/L), and

German Shepherd dogs (1.38 x 10^9/L) compared to the overall population (0.91 x 10^9/L). Recently, dogs from a small size breed (i.e., D) had higher mean PCV (52% *versus* 50%), mean RBC count (7.7 *versus* 7.1 x 10^{12}/L) and HBG concentrations (18.2 *versus* 16.8 g/dL) compared to a mixed-breed population *(Torres et al., 2014)*. Finally, a report *(Lavoué et al., 2014)* emphasized that Dogue de Bordeaux exhibited higher RI compared to the standard laboratory's RI regarding a lot of hematological variables including HGB (12.8-20.6 *versus* 12.4-19.2 g/dL), PCV (35-56% *versus* 35-52%), MCV (22.6-26.1 *versus* 21.9-26.3 pg) and MCHC (347-382 *versus* 344-381 g/L). To the contrary, this molossoid type breed exhibited lower RI for reticulocyte (13.7-128.0 *versus* 19.4-150.1 x 10^9/L) and PLT (87-328 *versus* 108-562 x 10^9/L) counts compared to the generic RI.

2. Biochemistry

As early as in 1969, a breed effect on serum biochemistry was reported in Beagle dogs (*Cramer et al., 1969; Pickrell et al., 1974; Craig et al., 2006).* As for hematological variables, this effect has been more commonly described in Greyhounds (*Zaldívar-López et al., 2011; Lefebvre, 2011).* In one previous study including 30 adult healthy Greyhounds, the mean serum creatinine concentration (1.6 mg/dL, range 1.2-1.9 mg/dL) in this breed was significantly higher than that in 30 adult healthy non-Greyhound dogs (1.03 mg/dL, range 0.8-1.7 mg/dL), (**Figure 9**, *Feeman et al., 2003*). Besides, in 14 out of the 30 Greyhounds (46.6%), the serum creatinine concentration was above the RI established in the general canine population.

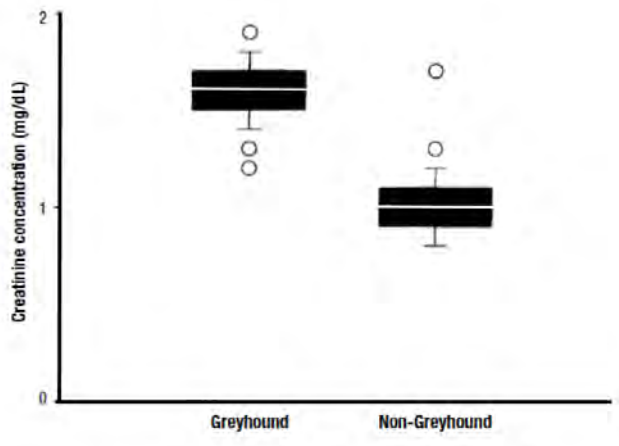

Figure 9. Serum creatinine concentration in Greyhound *versus* non-Greyhound dogs. *The horizontal white bar represents the median value. The upper and lower limits of the box represent the 25th and 75th percentiles. The whiskers represent the 10th and 90th percentiles. The rounds represent extreme values. From Feeman et al. Serum creatinine concentrations in retired racing Greyhounds. Vet Clin Pathol 2003;32:40-42.*

Another study established RI for 12 biochemical variables in a population of 499 healthy Greyhound blood donors. The results showed that RI for total protein, albumin, globulin and creatinine differed from the generic RI (*Dunlop et al., 2011)*. The difference was important mainly for creatinine (RI in Greyhounds: 99-174 µmol/L *versus* 20-150 µmol/L in non-Greyhounds), according to previous studies *(Feeman et al., 2003; Steiss et al., 2000).* The low serum total protein value in Greyhounds was explained here by low concentrations of alpha- and beta-globulins demonstrated in another study *(Fayos et al., 2005).* Other authors reported that basal serum total and free-thyroxine concentrations were significantly lower in Greyhounds than in non-Greyhounds, even after thyroxine stimulating and releasing hormones administration *(Gaughan and Bruyette, 2000).* Same results were found regarding serum total thyroxine concentration in Basenjis *(Seavers et al., 2008).*

Regarding other breeds, one study *(Sharkey et al., 2009)* evaluated a total of 16 serum biochemical analytes among dogs from 4 different healthy adult large breeds (59 Alaskan Malamutes, 78 Siberian Huskies, 90 Golden Retrievers and 77 English Setters). The authors found statistically significant differences within the breeds for most of the tested variables **(Table 10)**. However, the authors stated that there was a large degree of overlap in analyte ranges within the different breeds. More interestingly, the between-breed differences for many analytes were within the analytical imprecision of the assays.

Table 10. Pairwise comparison between breeds indicating significant differences (X). *AM, Alaskan malamute; SH, Siberian Huskies; ES, English Setters and GR, Golden retrievers. From Sharkey et al., Breed-associated variability in serum biochemical analytes in four large-breed dogs. Vet Clin Pathol 2009;38:375-380.*

	Breed Comparison					
Analyte	AM vs SH	AM vs GR	AM vs ES	SH vs GR	SH vs ES	GR vs ES
Urea						
Creatinine	X		X	X		X
Calcium		X				X
Phosphorus					X	X
Magnesium	X	X			X	X
Total protein	X	X	X	X		
Albumin	X			X	X	
Globulin	X	X	X			
Sodium			X	X	X	X
Potassium	X	X		X		X
Bicarbonate	X		X	X	X	X
Bilirubin						
γ-Glutamyltransferase				X		X
Alanine aminotransferase	X	X	X	X	X	X
Aspartate aminotransferase	X		X			X
Amylase	X	X	X	X	X	

Among 11 evaluated plasma biochemistry variables in 62 healthy Shetland sheepdogs *(Sato et al., 2000),* the plasma cholesterol concentration was significantly higher (mean±SD: 333±311 mg/dL) in Shetlands than in healthy control dogs from other breeds (191±79 mg/dL).

Additionally, in Bernese Mountain dogs *(Nielsen et al., 2010)*, RI derived from the general canine population regarding the majority of 18 tested biochemical variables were readily applicable. However, for 5 analytes (alkaline phosphatase (ALP), gamma-glutamyl-transferase, albumin, cholesterol and total bilirubin), significant deviations were observed, requiring *de novo* establishment of new RI within the breed.

Moderate to marked differences were observed between RI in Dogue de Bordeaux and generic RI for 6 biochemical variables *(Lavoué et al., 2013)*. Hence, as compared to the manufacturer's RI, a marked downward shift for total thyroxine and upward shifts for lipase, activities of aspartate (AST) and alanine (ALT) aminotransferases, as well as for total proteins and cholesterol concentrations were observed. The most significant difference was identified for lipase activity, for which the RI of 75% of Dogues de Bordeaux was largely above the usual RI.

Among 96 North American Scottish Deerhounds (*Sheerer et al., 2013)*, most of RI were similar to the generic RI, although narrower. An exception was the total calcium concentration, for which more than 50% of the studied population was below the nonbreed-specific RI.

Finally, a breed effect has been identified for more specific analytes in dogs such as lipoproteins *(Downs et al., 1993),* C-reactive protein (*Wong et al., 2011),* immunological and coagulation markers (*Gerber et al., 2010; Nikolic Nielsen et al., 2011),* neuro-hormones such as plasma renine, aldosterone (*Pedersen et al., 1995b)* and endothelin *(Moesgaard et al., 2007b)*, as well as hormones involved in calcium homeostasis *(Tryfonidou et al., 2003).*

II.2.B. EFFECT OF AGE

A report including young, healthy, pre-training Greyhounds demonstrated that from the age of 9 to 10 months, Greyhounds had higher PCV, HGB concentration and RBC counts compared to adults *(Shiel et al., 2007)*. These differences were less marked in Greyhounds from 5 to 6 months old. Moreover, in the same study, the platelet count correlated negatively with age *(Shiel et al., 2007).* In a study including 68 puppies subjected to blood samples at different ages (i.e., 4, 10, 12, 16, 28, 70, 77 and 84 days), HCT, HGB, and glucose were significantly different at all ages (except at day 4) compared to adults for several variables such as sodium, potassium, chloride, ionized calcium and magnesium *(O'Brien et al., 2014).* Same observations were found in a population of 896 dogs <1 year old, where age significantly affected plasma albumin, ALT, ALP, blood urea nitrogen (BUN), glucose, lipase, and total protein *(Sako et al., 2011).* The most relevant effect of age in the later study was for ALP activity, with a mean of 471 UI/L in dogs aged between 4 and 5 months and 231 UI/L in dogs aged between 10 and 11 months. In Labradors and Beagles *(Harper et al., 2003),* RBC counts, HGB concentration, and PCV increased during the first year of life whereas WBC count decreased with increasing age. In young Beagles < 8 weeks and <1 year, median WBC count was, respectively, 19.0 x 10^9/L and 9.9 x 10^9/L *(Harper et al., 2003)*. In the same report *(Harper et al., 2003),* plasma ALP activity, total protein and total calcium were higher in the first years of life, according to a previous study in Beagles *(Ikeuchi et al., 1999),* whereas plasma phosphorus decreased progressively. Another report in the same breed showed that young beagles had a different renal function compared to older dogs and characterized by a higher daily urinary volume, glomerular filtration rate, free water reabsorption, a lower daily protein excretion, and fractional excretion of phosphorus

(Laroute et al., 2005). One report found that WBC, lymphocyte, and neutrophil counts decreased significantly with age, the most marked decrease being for the lymphocyte count, which decreased by about 50% between the ages of 1 year and 9-10 years (*Bourgès-Abella et al., 2011).* However, in Borzoï and Beagle puppies, there was no significant difference between puppies and adults regarding ALT activity, glucose concentration, MCHC, WBC and platelet counts *(Rosset et al., 2012).* Conflicting results were also found regarding the effect of age on hematological and biochemical variables in adult Dogue de Bordeaux *(Lavoué et al., 2013 & 2014).* More specifically, mean creatine kinase activity was 2 to 5 times higher in young animals up to one year old, after which there was no more significant effect of age (*Aktas et al., 1994)*. Additionally, mean serum α-1-acid glycoprotein concentration (i.e., an acute-phase protein and a serum marker of inflammation and neoplasia in humans) was lower in newborns (122 mg/L) and gradually increased to adult levels (364 mg/L) by 3 months of age *(Yuki et al., 2010).* Finally, in one study, effect of age on thyroid and adrenocortical hormones was also pointed out *(Reimers et al., 1990).*

II.2.C EFFECT OF GENDER

Limited data are available in the literature regarding the effect of gender on blood variables. Moreover, as for age, the results remain conflicting. In a population of dogs from different breeds, several authors found no effect of gender on biochemical variables such as creatine kinase *(Aktas et al., 1994)*, serum α-1-acid glycoprotein concentration *(Yuki et al., 2010)* and thyroid hormones *(Reimers et al., 1990).* Same observations were found in reports including a specific

breed or breed category. Hence, in Greyhounds *(Shiel et al., 20017; Dunlop et al., 2011)* and Dogue de Bordeaux *(Lavoué et al., 2013 & 2014),* methods to investigate the differences between male and female regarding hematological and biochemical RI did not provide any evidence supporting data partitioning by gender. To the contrary, a study including Greyhounds found that partitioning according to gender was statistically recommended for 8 out of 10 hematological variables except PCV and WBC, which fell into the grey zone, leaving the decision to non-statistical considerations *(Campora et al., 2011a).* In a population of 132 healthy purebred dogs *(Bourgès-Abella et al., 2011),* gender was a partitioning factor only for PCV and PLT counts, and separate RI for males and females were established. Finally, in young dogs (<1 year old), gender significantly influenced ALP activity as well as amylase, lipase, and total cholesterol concentrations *(Sako et al., 2011).*

II.2.D. OTHER EFFECTS

Numerous other physiological effects may influence the generation of a RI although some of them remain unknown in dogs. Exercise has been the most documented effect in dogs, especially in Greyhounds and sled dogs. Training or exercise in sled dogs may induce significant increase in sodium, chloride, albumin, calcium, and cortisol concentrations *(Angle et al., 2009)* as well as a decrease in PCV and an increase in WBC count *(Davis et al., 2008).* In Greyhounds, an increase in PCV immediately after racing that return to baseline in few hours has been demonstrated *(Horvath et al., 2014).* Additionally, a study including 15 dogs subjected to an agility test showed that this competition induced mild to moderate changes in hematologic and biochemical results

consistent with splenic contraction, increased lipolysis, and utilization of anaerobic pathways involved in energy re-synthesis in muscles *(Rovira et al., 2007)*. However, in untrained Beagle dogs, strenuous exercise did not induce major variations in any of plasma routine plasma variables *(Chanoit et al., 2002)*. Diet is also known to affect few biochemical and hematological variables in dogs, particularly the cholesterol concentration *(Swanson et al., 2004)*. In one previous study, obesity influenced plasma leptin concentration *(Ishioka et al., 2007)*. Finally, other effects such as pregnancy, lactation and neutered status may modify blood variables in dogs, but warrant investigations.

II.3. CONCLUSION

In conclusion, most of the studies within the literature agree that the influence of breed on hematological and biochemical variables exists in the canine species. However, results are more conflicting regarding the effect of age and gender and also the influence of BW, which is often confounded by the breed. Some of these effects may be significant, as shown for the creatinine values in Greyhounds compared to non-Greyhounds or the ALP activity in young compared to adult dogs. However, some differences may have little impact on the clinical interpretation of the results, as remarkably pointed out in the study by *Sharkey et al (2009),* where the differences between breeds for most of the biochemical analytes were within the analytical imprecision of the assays. Finally, numerous other effects may or may not influence blood variables in dogs but remain unknown.

III. REFERENCE INTERVAL IN VETERINARY CARDIOLOGY

III.1. INTRODUCTION

To date, no RI according to the IFCC-CLSI guidelines have been published in veterinary cardiology. However, a lot of studies focused on the evaluation of heart morphology and function in healthy dogs by using radiography **(Table 11)**, electrocardiography (ECG, **Table 12**), cardiac biomarkers **(Table 13)** and echocardiography **(Table 14)**. Moreover, heart evaluation, especially by using TTE, has been performed in healthy individuals within other animal species including cats *(Kayar et al., 2014; Chetboul et al., 2006 & 2012b; Mottet et al., 2012),* horses *(Blissit and Bonagura, 1995; Grenacher and Schwarzwald, 2010)*, cattle *(Hallowell et al., 2007; Buczinski, 2009),* goats *(Leroux et al., 2012),* pigs *(Konrad et al., 2000)*, ferrets *(Malakoff et al., 2012)*, rabbits *(Casamian-Sorrosal et al., 2014)*, hares *(Noszczyk-Nowak et al., 2009),* dolphins *(Sklansky et al., 2006; Chetboul et al., 2012c)*, chimpanzees *(Sleeper et al., 2014),* gorillas *(Murphy et al., 2011),* manatees *(Gerlach et al., 2013),* camels *(Tharwat et al., 2013),* terrapins *(Poser et al., 2011),* hedgdogs *(Black et al., 2011),* wolves *(Estrada et al., 2009),* zebras *(Adin et al., 2007),* psittacine birds *(Pees et al., 2004)* and birds of prey *(Straub et al., 2003)* and grizzly bears *(Nelson et al., 2003).*

Table 11. Studies about radiographic evaluation of heart in healthy dogs. *CKCS, Cavalier King Charles Spaniel; SD, Standard deviation.*

Authors	Breed	Number of dogs	Age	Body weight	Variable	Data provided
Lamb et al., 2001	Boxer, Labrador, Doberman, CKCS, Yorkshire Terrier, German Shepherd	107	Range: 1.0-15.0 years	Data not shown	Vertebral heart scale	Mean±SD and 95% confidence interval
Sleeper et al., 2001	Golden and Labrador Retrievers, Beagle and German Shepherd puppies	11	Range: 3.0-36.0 months	Range: 5.3-10.0 kg	Vertebral heart scale	Mean±SD
Bavegems et al., 2005	Whippet	44	Range: 11.0-142.0 months	Range: 9.3-17.2 kg	Vertebral heart scale	Mean±SD
Marin et al., 2007	Greyhound	42	Data not shown	Data not shown	Vertebral heart scale	Mean±SD
Kraetschmer et al., 2008	Beagle	19	Range: 3.0-5.0 years	Range: 14.5-22.2 kg	Vertebral heart scale	Mean±SD, range
Jepsen-Grant et al., 2012	Pug, Pomeranian, Yorkshire Terrier, Dachshund, Bulldog, Shih-Tzu, Lhassa Apsos, Boston Terrier	204	Range: 1.5-15.5 years	Data not shown	Vertebral heart scale	Mean±SD

Table 12. Studies about electrocardiography in healthy dogs. *CKCS, Cavalier King Charles Spaniel; SD, Standard deviation.*

Authors	Breed	Number of dogs	Age	Body weight	Variable	Data provided
Eckenfels and Trieb, 1979	Beagle	432	Range: 187-646 days	Range: 5.8-18.5 kg	Electrocardiography and heart rate	Range
Tilley, 1992	Data not shown	Data not shown	Data not shown	Data not shown	Electrocardiography	Range
Calvert et al., 1998	Doberman	55	Range: 1-13 years	Data not shown	Electrocardiography (signal average)	Mean±SD, Mean±2SD, range
Hanton and Rabemampianina, 2006	Beagle	1880	Range: 13.0-20.0 months	Data not shown	Electrocardiography	Mean±SD, range
Bavegems et al., 2009	Whippet	105	Range: 10.0-169.0 months	Range: 9.3-17.2 kg	Electrocardiography	Median and interquartile range
Rasmussen et al., 2011	CKCS, Dachshund, Cairn Terrier	50	Range: 2.0-9.0 years	Data not shown	Holter and heart rate	Mean and range

Table 13. Studies about cardiac biomarkers in healthy dogs. *CKCS, Cavalier King Charles Spaniel; SD, Standard deviation.*

Authors	Breed	Number of dogs	Age	Body weight	Variable	Data provided
Eriksson et al., 2001	CKCS	17	Range: 5.0-9.5 years	Range: 6.6-12.0 kg	N-terminal pro-atrial-type, brain natriuretic peptides, endothelium derived relaxing factor, nitrate, nitrite, endothelin-1, cyclic guanosine monophosphate	Mean±SD, range
Sleeper et al., 2001b	Mixed breed, Labrador retriever, American Pitt Bull Terrier, German Shepherd and other	41	Range: 5.0-10.0 years	Data not shown	Troponin I	Range, 90th percentiles with 95% confidence interval
Adin et al., 2005	Mixed breed, Boxer, Golden and Labrador Retrievers, Doberman and other	54	Mean±SD: 4.8±3.1 years	Mean±SD: 24.4±11.2 kg	Troponin I	5th and 95th percentiles
LaVecchio et al., 2009	Greyhound	20	Range: 5.0-9.0 years	Data not shown	Troponin I	Median, range, 25th and 75th percentiles
Kellihan et al., 2009	Mixed breed, Labrador Retriever, Keeshonds, American Staffordshire Terrier and other	53	Range: 0.8-10.0 years	Range: 6.8-50.0 kg	N-terminal pro-brain-type natriuretic peptide	Median, range, 25th and 75th percentiles
Sjöstrand et al., 2014	Labrador retriever, Belgian and German Shepherds, Finnish Lapphund and other	535	Median: 3.3 years	Median: 30.2 kg	N-terminal-pro-brain-type and pro-atrial-type natriuretic peptides	Median and interquartile range

Table 14. Studies about echocardiography in healthy dogs. *CKCS, Cavalier King Charles Spaniel; SD, Standard deviation; ST, speckle tracking imaging; TDI, tissue Doppler imaging; 2D, bidimensional.*

Authors	Breed	Number of dogs	Age	Body weight	Variable	Data provided
Boon et al., 1983	Beagle, Golden Retriever, Dingo, Doberman and other	20	Range: 0.5-6.0 years	Range: 9.8-28.6 kg	M-mode echocardiography	Mean±SD, range, 95% confidence interval
Lombard, 1984	Data not shown	40	Range: 1.0-9.0 years	Range: 5.0-44.0 kg	M-mode echocardiography	Mean±SD
Gooding et al., 1986	English Cocker Spaniel	17	Range: 2.0-7.0 years	Range: 8.5-16.3 kg	M-mode echocardiography	Mean±SD
O'Grady et al., 1986	Data not shown	18	Range: 0.8-5.5 years	Range: 5.5-30.0 kg	M-mode echocardiography	Mean±SD, range, regression equation
Yuill and O'Grady, 1991	Data not shown	20	Range: 1.5-8.0 years	Range: 3.0-41.0 kg	Standard Doppler	Mean±SD
Crippa et al., 1992	Beagle	50	Around 28 weeks	Mean: 8.9 kg	M-mode echocardiography	Mean±SD, range
Morrison et al., 1992	Miniature Poodle, Pembroke Welsh Corgi, Afghan Hound, Golden Retriever	80	Range: 2.0-7.0 years	Range: 1.4-41.0 kg	M-mode echocardiography	Median and range
Bayon et al., 1994	Spanish Mastiff	66	Range: 1.0-12.0 months	Range: 3.3-52.4 kg	M-mode echocardiography	Mean±SD
Snyder et al., 1995	Greyhound	11	Mean±SD: 5.5±2.5 years	Mean±SD: 25.0±36.3 kg	M-mode echocardiography	Mean±SD, range, regression equation
Page et al., 1996	Greyhound	16	Adult	Range: 20.7-32.5 kg	M-mode echocardiography	Mean±SD, range

Authors	Breed	Number of dogs	Age	Body weight	Variable	Data provided
Lonsdale et al., 1998	Greyhound	55	Range: 1.5-11.0 years	Range: 21.9-39.0 kg	M-mode echocardiography	Mean±SD
Vollmar et al., 1999	Irish Wolfhound	262	Range: 1.0-8.5 years	Range: 48.0-93.0 kg	2D and M-mode echocardiography	Mean±2SD
Della Torre et al., 2000	Greyhound, Whippet and Italian Greyhound	60	Range: 2.0-9.0 years	Range: 3.2-31.5 kg	2D, M-mode and standard Doppler echocardiography	Mean±SD
Gonçalves et al., 2002	Data not shown	69	Mean±SD: 4.1±2.6 years	Range: 3.9-97.7 kg	M-mode echocardiography	Equation for 95% predictive interval
Hansson et al., 2002	CKCS	56	Mean±SD: 5.2±2.6 years	Mean±SD: 8.7±1.4 kg	2D and M-mode echocardiography	Mean±SD, range
Brown et al., 2003	Golden and Labrador Retrievers, Great Dane, mixed-breed	53	Median: 5.3; Range: 0.12-12.7 years	Median: 24.6 Range: 2.3-76.4 kg	M-mode echocardiography	Ratio indices based on aortic root or weight-based aortic root
O'Leary et al., 2003	Bull Terrier	14	Range: 9.0-30.0 months	Range: 18.0-32.0 kg	2D, M-mode and standard Doppler echocardiography	Equation for 95% predictive interval
Cornell et al., 2004	CKCS, Dachshund, Boxer, Irish Wolfhound and other	494	Data not shown	Range: 2.2-95 kg	M-mode echocardiography	Linear regression analysis and prediction intervals
Baumwart et al., 2005	Data not shown	45	Range: 0.6-8.0 years	Range: 3.0-55.0 kg	Tei index of right myocardial performance	Mean±SD and 95% confidence interval

Authors	Breed	Number of dogs	Age	Body weight	Variable	Data provided
Chetboul et al., 2005	Beagle, German Shepherd, Belgian Malinois, Golden Retriever and other	100	Range: 0.4-8.8 years	Range: 6.0-49.0 kg	2D, M-mode, standard Doppler and Tissue Doppler imaging echocardiography	Mean±SD, range
Hetyey et al., 2005	Irish Wolfhound, Bull Mastiff, Caucasian and German Shepherds and other	20	Range: 0.5-13.0 years	Range: 1.0-60.0 kg	2D echocardiography	Raw data
Kayar et al., 2006	German Shepherd	50	Data not shown	Range: 28.0-40.0 kg	2D and M-mode echocardiography	Mean±SD
Muzzi et al., 2006	German Shepherd	60	Range: 1.0-5.0 years	Range: 22.0-37.0 kg	2D, M-mode and standard Doppler echocardiography	Mean±SD
Bavegems et al., 2007	Whippet	125	Mean±SD: 4.9±3.2 years	Mean±SD: 13.2±2.1	2D, M-mode and standard Doppler echocardiography	Mean±SD, range, mean±2SD
Cunningham et al., 2008	Boxer	81	Median: 6.1; Range: 2.1-11.0 years	Median: 28.9; Range: 18.9-40.5 kg	2D, M-mode and standard Doppler echocardiography	Median, range and weight-based ratio indices
Lobo et al., 2008	Estrela Mountain dog	74	Range: 18-123 months	Range: 30.0-75.0 kg	2D, M-mode and standard Doppler echocardiography	Mean±SD, mean±2SD
Serres et al., 2009b	CKCS, mixed breed, Labrador and Golden Retrievers and other	50	Range: 0.8-13.0 years	Range: 1.8-52.0 kg	2D and Doppler echocardiography (pulmonary to systemic flow ratio)	Mean±SD, range
Vöros et al., 2009	Hungarian Vizla, Mudi and Hungarian Greyhounds	95	Range: 6.0-10.0 years	Range: 12.5-32.5 kg	2D and M-mode echocardiography	Mean±SD, range

Authors	Breed	Number of dogs	Age	Body weight	Variable	Data provided
Diez-Prieto et al., 2010	Beagle	6	4 to 21 months	7.2-10.9 kg	2D, M-mode and standard Doppler echocardiography	Mean±SD at 4, 7, 10, 13, 17 and 21 months
Griffiths et al., 2011	Shih-Tzu, Boxer, Tornjak, Greyhound, Beagle	10	Range: 1.5-7.0 years	Range: 5.5-41.7 kg	ST echocardiography (ventricular synchrony)	Mean±SD, range
Locatelli et al., 2011	Dogue de Bordeaux	31	Mean±SD: 30.1±7.8 months	Mean±SD: 51.2±6.5 kg	2D, M-mode and standard Doppler echocardiography	Range and regression equation
Simak et al., 2011	Doberman	100	Mean±SD: 6.3±1.9 years	Mean±SD: 36.2±4.6	ST echocardiography and TDI	Mean±SD, range
Pariaut et al., 2012	Data not shown	50	Data not shown	Mean±SD: 16.0±10.5 kg	M-mode echocardiography (triscuspid annular plane systolic excursion)	Range, 2.5th and 97.5th percentiles
Stephenson et al., 2012	Great Dane	40	Data not shown	Data not shown	M-mode and 2D echocardiography	Median, 2.5th and 97.5th percentiles
Höllmer et al., 2013	Chihuahua, Border Terrier, Dachshund, CKCS and other	237	Range: 1.0-15.4 years	Range: 1.9-92.0 kg	2D echocardiography	Mean±SD
Jacobson et al., 2013	Border Collie	20	Range: 2.0-12.0 years	Range: 15.0-29.0 kg	2D, M-mode and standard Doppler echocardiography	Mean±SD, range, 95% confidence interval
Westrup and McEvoy, 2013	Irish Wolfhound	46	Range: 1.0-8.2 years	Range: 53.0-85.0 kg	ST echocardiography	Mean±SD
Smets et al., 2014	Boxer	85	Range: 1.0-14.5 years	Mean±SD: 29.8±4.6 kg	Conventional echocardiography (ejection fraction of left ventricle)	Mean±SD

III.2. EFFECT OF COVARIATES ON CARDIOLOGIC VARIABLES IN DOGS

III.2.A. EFFECT OF BODY WEIGHT

1. Radiographic and echocardiographic variables

The effect of BW on heart size assessed by thoracic radiographs and TTE has been demonstrated in many studies in dogs because size of the heart is strongly correlated to BW *(Lombard et al., 1984; Sisson and Schauffer, 1991)*. In the radiographic assessment of the heart, determination of VHS has been proposed to encompass the BW effect *(Buchanan and Bücheler, 1995)*. Hence, VHS allows assessment of lengths of long and short axis of the heart scaled against the length of vertebrae dorsal to the heart (i.e., a BW-dependent variable). Concerning TTE, all studies **(Table 14)** agree that a BW effect exists on echocardiographic variables, especially for M-mode measurements of left ventricle diameter and wall thickness, Ao and LA. Therefore, authors have developed different mathematical equations from general canine populations to predict M-mode RI according to BW *(Lombard et al., 1984; O'Grady et al., 1986; Jacobs and Mahjoob, 1988; Morrison et al., 1992; Gonçalves et al., 2002; Cornell et al., 2004),* body surface area (*Boon, 1983)* and aortic root dimension *(Brown et al., 2003).* As an example, according to *Cornell et al., 2004*, the 95% prediction interval of LVED is 19.0-28.0 mm and 38.0-55.0 in dogs weighing respectively 4 and 40 kg. Similarly, these methods have been applied to specific breeds, as described in Spanish Mastiffs *(Bayon et al., 1994),* Greyhounds *(Snyder et al., 1995),* Bull Terriers *(O'Leary et al., 2003)*, Boxers *(Cunningham et al., 2008)* and Dogues de Bordeaux

(Locatelli et al., 20011). While considering Doppler echocardiography, a BW effect was reported but considered modest by the authors for pulmonary and aortic velocities assessed by continuous wave Doppler *(Brown et al., 1991; Yuill and O'Grady, 1991).* Additionally, no significant BW effect was observed for isovolumic relaxation time *(Schober and Fuentes, 2001),* trans-mitral *(Yuill and O'Grady, 1991; Schober and Fuentes, 2001)* or trans-tricuspid flows *(Yuill and O'Grady, 1991).*

2. *Electrocardiographic variables*

Studies on ECG and Holter examination did not find any BW effect in Beagles *(Eckenfels and Trieb, 1979; Hanton and Rabemampianina, 2006),* Dobermans *(Calvert et al., 1998)* and Whippets *(Bavegems et a., 2009)*, as well as in CKCS, D and Cairn Terriers *(Rasmussen et al., 2011).*

3. *Cardiac biomarkers*

The effect of BW on cardiac biomarkers has been poorly investigated. Most of the studies did not identify any age or BW effect on the biomarker NT-proBNP *(Boswood et al., 2008; Tarnow et al., 2009; Kellihan et al., 2009; Oyama et al., 2008; Ettinger et al., 2012).* However, one study performed in 539 healthy dogs from 9 different breeds including CKCS found that NT-proBNP, but not pro-ANP concentration, was significantly increased with increasing BW *(Sjöstrand et al., 2014).* To the contrary, NT-proBNP was inversely related to BW in a group of 39 asymptomatic CKCS with DMVD *(Tarnow et al., 2009).* Another study identified a positive correlation

between NT-proBNP and BW in a group of 40 healthy control dogs *(Oyama et al., 2008).* In CKCS, NT-proANP concentration decreased with increasing BW, whereas no effect was found regarding the BNP *(Eriksson et al., 2001).* Nevertheless, although a correlation was identified, the magnitude of BW effect on cardiac biomarker concentration in dog was not investigated.

III.2.B. EFFECT OF BREED

1. Radiography and echocardiography

In 1992, *Morrison et al.* performed a study on M-mode echocardiographic measurements including 80 healthy dogs from 4 different breeds and somatotypes (Pembroke Welsh Corgi, MP, Afghan Hound and Golden Retriever). Interestingly, the results showed that for 10 out of 11 M-mode variables, means were significantly different between all breeds after the differences in BW were taken into account. The authors concluded therefore that not only the BW, but also the breed, must be taken into account while establishing echocardiographic RI. Soon after, numerous studies started to illustrate breed effect in veterinary cardiology. Concerning heart radiography, one study *(Lamb et al., 2001)* reported that normal Boxer dogs had significantly higher VHS mean value (i.e., 11.6) compared to 3 other large breeds (e.g., mean VHS: 9.7 in German Shepherd, 10.0 in Dobermans and 10.8 in Labradors). The same observation was made between YT and CKCS (mean VHS: 9.7 *versus* 10.6, in YT and CKCS respectively) and between Greyhounds and other breeds *(Bavegems et al., 2005; Marin et al., 2007).* Another study *(Jepsen-Grant et al., 2013)* showed that brachycephalic dogs (Pugs, Bulldogs, Boston Terriers) and

Pomeranians had significantly higher VHS compared to the previously published RI *(Buchanan and Bücheler, 1995).*

Considering echocardiography, sighthound dogs have again been the most commonly studied breed category. Greyhounds have been reported with an increased heart weight to BW ratio and gross evidence of myocardial thickening as well as chamber dilation compared to other breeds *(Schneider et al., 1964; Steel et al., 1976).* These observations were further demonstrated in this breed by using TTE *(Page et al., 1993; Snyder et al., 1995)* and were more striking in trained *versus* non-trained dogs *(Lonsdale et al., 1998).* The same findings were emphasized in 105 healthy adult Whippets *(Bavegems et al., 2007).* Regarding LVED and LVES measurements, 29.6% and 35.2% of Whippets were above the predictive value established by *Cornell et al., 2004.*

One study reported that several healthy English Cocker Spaniels had low fractional shortening but the reason of such a finding remained unclear *(Gooding et al., 1986)*. Moreover, compared to other breeds, German Shepherds *(Muzzi et al., 2006)* and Border Collies *(Jacobson et al., 2013)* had larger left ventricle diameter, whereas Bull Terriers *(O'Leary et al., 2003)* and Boxers *(Cunningham et al., 2008)* exhibited larger left ventricular wall thicknesses, LA diameter, greater aortic velocity and smaller aortic root size.

2. <u>*Electrocardiography*</u>

A study showed that ECG characteristics in Whippets were similar to those reported in human athletes *(Bavegems et al., 2007).* Regarding some parameters (P-wave amplitude, ST-segment

deflection and T-wave amplitude, R-wave amplitude), a marked percentage (i.e., 21 to 81%) of the Whippet values were above the published maximum reference limits established in other dogs. The most important difference was for the median R-wave amplitude, which was higher in Whippets and could have been considered as pathological in such a dog when compared to a non-Whippet RI. Additionally, a study in small size dogs (including CKCS and D) showed that 15 out of 27 Holter-derived variables were significantly influenced by the breed *(Rasmussen et al., 2011)*.

3. Cardiac biomarkers

The breed effect on troponin I concentration has only been evaluated within Greyhounds. In one previous study *(LaVecchio et al., 2009)*, retired racing Greyhounds had significantly higher median troponin I concentration (0.08 ng/mL) compared to a non-Greyhound group (0.02 ng/mL), requiring specific RI establishment. However, no difference was found between Greyhounds and a group of healthy Boxer dogs **(Figure 10)**.

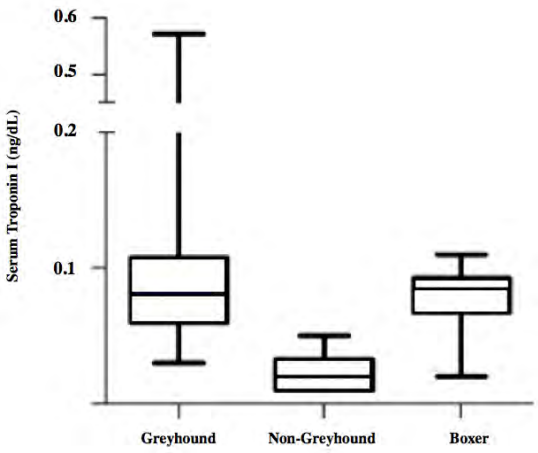

Figure 10. Box-and-whisker plot depicting the serum troponin I concentrations in healthy Greyhound, non-Greyhound and Boxer dogs. *The horizontal bar represents the median value and the whiskers represent the 2.5% and 97.5 percentiles. From LaVecchio et al. Serum cardiac troponin I concentration in retired racing greyhounds. J Vet Intern Med 2009;23:87-90.*

Considering natriuretic peptides, a recent study involving 535 healthy dogs from 9 different breeds showed that significant differences exist between breeds *(Sjöstrand et al., 2014)*. Plasma proANP concentration was lowest in Doberman Pinschers, with a median concentration almost half of the median values in German Shepherds and CKCS, which had the highest concentrations **(Figure 11A)**. Moreover, concentrations of plasma NT-proBNP were lowest in D, whereas Labrador Retrievers and Newfoundlands had the highest concentrations with median values 3 times the median value in D **(Figure 11B).**

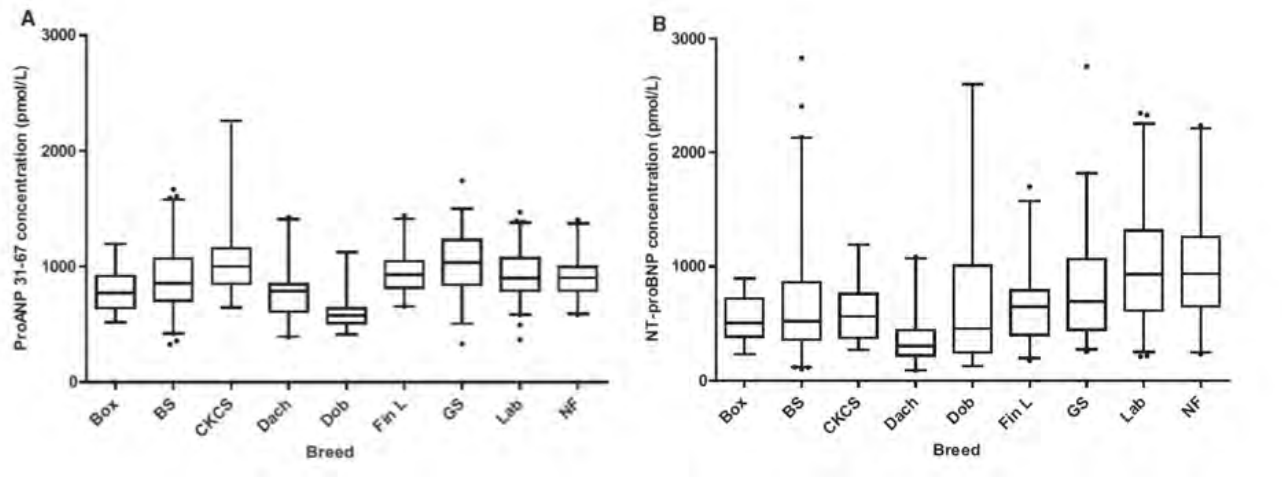

Figure 11. Boxplots showing distribution of the natriuretic peptides pro-atrial-type (ANP 31-67, Figure 11A) and N-terminal pro-brain-type (NT-proBNP, Figure 11B) by breed. *The top, bottom, and line through the middle of each box correspond to the 75th percentile (top quartile), the 25th percentile (bottom quartile) and the 50th percentile (median), respectively. The whiskers extend from the bottom 2.5th percentile to the top 97.5th percentile. Outliers are represented by black dots. Box, Boxer; BS, Belgian Shepherd; CKCS, Cavalier King Charles Spaniel; Dach, Dachshund; Dob, Doberman Pinscher; Fin L, Finnish Lapphund. From Sjöstrand et al. Breed differences in natriuretic peptides in healthy dogs. J Vet Intern Med 2014;28:451-457.*

III.2.C. EFFECT OF AGE

1. Radiography and echocardiography

In adult dogs, no significant effect of age on VHS has been demonstrated. Similarly, in growing puppies, although absolute measurements (e.g., lateral and dorso-ventral long and short axis of the heart) increase with age as expected, differences among VHS measurements at 3, 6 and 12

months were not significant *(Sleeper et al., 2001)*. Regarding TTE variables, the effect of age has been documented in several breeds or breed categories, but results remain conflicting, especially in adults. In growing Beagle aged between 4 and 21 months, a significant effect of age was demonstrated for variables reflecting heart size such as ventricular diameter and wall thicknesses. However, variables evaluating systolic and diastolic function were not affected by age *(Diez-Prieto et al., 2010).* In adult German Shepherds, no correlation was found between age and M-mode *(Kayar et al., 2006; Muzzi et al., 2006)* or Doppler *(Muzzi et al., 2006)* echocardiographic variables. However, in Dogues de Bordeaux >12 months old, age was positively associated with left ventricular wall thicknesses, left ventricular mass and right ventricular diameter (*Locatelli et al., 2011).* Additionally, increasing age resulted in a decrease in pulmonary velocity. Nevertheless, the differences were negligible as illustrated by the low slope coefficients found in the regression equation model. Hence, a difference in 5 years between 2 Dogues de Bordeaux will result in a 0.33 mm increase in right ventricular diameter, a 3.4 g increase in left ventricular mass and a 0.05 m/s decrease in maximal pulmonary velocity (*Locatelli et al., 2011).* Same results were found in Irish Wolfhounds *(Vollmar et al., 1999)* and Estrela Mountain dogs *(Lobo et al., 2008)* regarding several M-mode, bidimensional and Doppler variables. In 3 Hungarian breeds, a positive correlation was found between LA/Ao ratio and age, but the clinical relevance of such a finding was not investigated *(Vörös et al., 2009)*. Finally, an age effect was found concerning several Doppler variables reflecting left ventricle diastolic function. One study *(Schober et al., 2001)* reported that the isovolumic relaxation time was significantly higher in dogs > 13 years compared to young dogs <2 years and that mitral E wave decreased whereas A wave increased with increasing age, resulting in a decreased E/A ratio *(Schober et al., 2001)*. These findings were consistent with increasing ventricular stiffness, delayed relaxation and

altered diastolic function in old dogs, as previously described in human's elderly *(Gardin et al., 1998).*

2. Electrocardiography

In CKCS, D and Cairn Terriers, no effect of age was found on Holter variables (*Rasmussen et al., 2011)*. Other report studying ECG did not evaluate the age effect.

3. *Cardiac biomarkers*

Only few studies reported the effect of age on cardiac biomarkers and the clinical relevance of this effect remains unclear. In a study including 453 healthy Doberman Pinschers *(Wess et al., 2010)*, mean serum cardiac troponin I was significantly higher in dogs > 8 years (0.22 ng/mL) compared to younger dogs (0.12 ng/mL in dogs between 6-8 years and 0.03 ng/mL in dogs between 2-4 years). Additionally, plasma NT-proBNP concentration was significantly increased in healthy Doberman Pinschers > 8 years as compared with younger dogs (median: 295 *versus* 395 pmol/L) from the same breed *(Wess et al., 2011).* In CKCS, only plasma NT-proANP was positively associated with age, as shown in **Figure 12** *(Eriksson et al., 2001).*

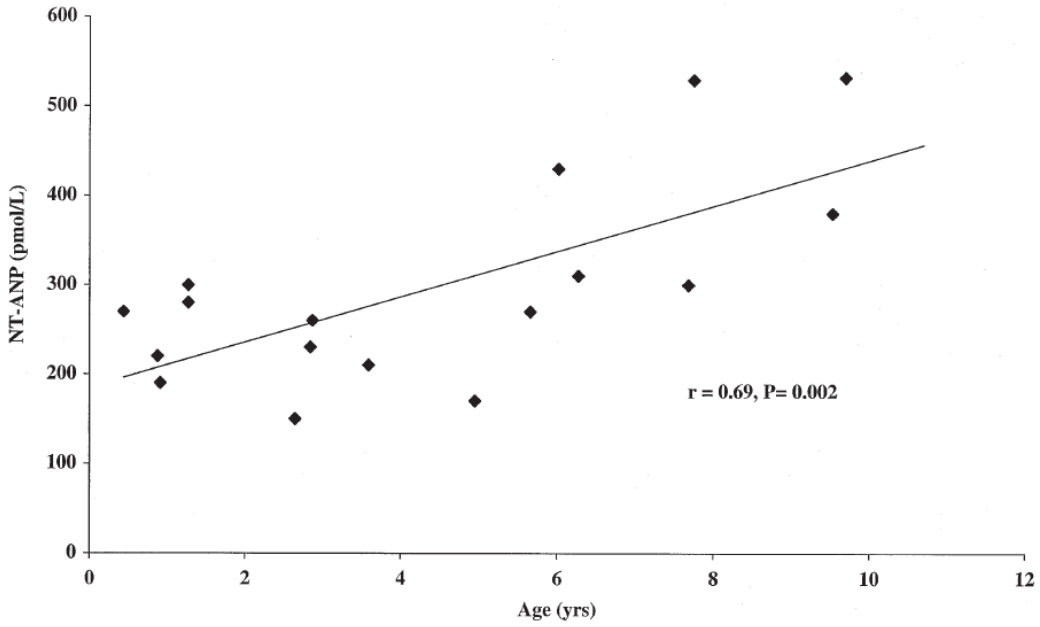

Figure 12. Linear regression plot for plasma N-terminal atrial-type natriuretic peptide (NT-ANP) concentrations in 17 healthy Cavalier King Charles Spaniels. *The regression formula is Y = 184.9 + 25.4x with Y, the NT-ANP concentration and x, the age of the dogs. From Eriksson et al. Effect of age and body weight on neurohumoral variables in healthy Cavalier King Charles spaniels. Am J Vet Res 2001;62:1818-1824.*

III.2.D. EFFECT OF GENDER

1. Radiography and echocardiography

The VHS in Beagle dogs was not different according to gender *(Kraetschmer et al., 2008).* However, in a study including 6 different small and large breed dogs (*Lamb et al., 1991)*, as well

as in Greyhounds (*Marin et al., 2007),* females had smaller VHS than males, but the magnitude of such a result was not investigated.

Concerning echocardiography, a gender effect has been reported in several studies whereas no effect was observed in Irish Wolfhounds *(Vollmar et al., 1999),* Hungarian dogs *(Vörös et al., 2009)* and Border Collies *(Jacobson et al., 2014).* In German Shepherds, one study did not find any gender effect on echocardiographic variables (*Kayar et al., 2006)*, whereas another report found a significant difference between males and females only for thicknesses of left and right ventricular walls *(Muzzi et al., 2006).* In Beagles *(Crippa et al., 1992)*, among 10 M-mode variables, only the left ventricular free wall (in both systole and diastole) was higher in males than in females (14.3% and 21.0% higher, respectively). In Whippets, although no significant difference in BW was observed between males and females, the later had larger left ventricular dimension, higher E-point to septal separation and also higher pulmonary velocity flow than males *(Bavegems et al., 2007)*. In Dogues de Bordeaux, only the end-diastolic left ventricular free wall thickness was influenced by gender but the difference was not relevant according to the low slope coefficient in the regression equation (i.e., 0.024). Finally, in Estrela Mountain dogs *(Lobo et al., 2008)*, a significant effect of gender was demonstrated for interventricular septum thickness at end-systole and left ventricular diameter at end-diastole, leading to the determination of separate RI for these variables. However, there was a large overlap between RI in males and females.

2. Electrocardiography

In Beagle *(Hanton et al., 2006)*, CKCS, D and Cairn Terrier dogs *(Rassmusen et al., 2011)*, no major differences in ECG variables between males and females. In Whippets *(Bavegems et al., 2009)*, males had significantly larger R wave amplitude than females (4.58 *versus* 4.13 mV, respectively). This difference, however, was no longer significant when the model was adjusted for weight and age (P = 0.28).

3. Cardiac biomarkers

No study identified a gender effect on the cardiac troponin I concentration. Regarding other natriuretic peptides, a recent report including 116 healthy control dogs found an effect of gender and neutered status *(Wolf et al., 2013),* with intact females having higher mean plasma NT-proBNP and NT-proANP values than intact males (i.e., 593 *versus* 315 pmol/L and 1036 *versus* 836 fmol/L, respectively). However, when considering only spayed dogs, no significant effect of gender was observed *(Wolf et al., 2013)*.

III.2.E. OTHER EFFECTS

In sled dogs, the effect of endurance training has been reported to modify ECG variables such as decrease heart rate, increase of QRS duration and QT-interval prolongation, reflecting exercise-

induced cardiac hypertrophy also called athletic heart syndrome *(Constable et al., 1994 and 2000)*. This syndrome has been described by using TTE in sled dogs and Greyhounds, with an eccentric hypertrophy of the left ventricle *(Lonsdale et al., 1998; Stepien et al., 1998)*. Pregnancy is also known to induce mild echocardiographic *(Abbott, 2010)* changes in healthy bitches such as increased FS (%), pulmonary and aortic ejection velocities and mitral E-wave. Regarding body condition, left ventricular hypertrophy and diastolic dysfunction have been documented in several obese dogs *(Mehlman et al., 2013)*. Finally, a recent study showed that emotional stress affected peak aortic velocity in healthy Boxers and Boxers with sub-aortic stenosis *(Pradelli et al., 2014)*.

III.3. CONCLUSION

A consensus between studies exists regarding the effect of BW on echo-Doppler variables. Moreover, the breed should be taken into account when establishing such RI because canine breeds may have particularities, as demonstrated in athletic breeds such as the Greyhound. However, the effect of age and gender is more controversial and considered clinically irrelevant in most of the cases. Regarding ECG, radiography and cardiac biomarkers, only few studies investigated the effect of physiological covariates such as breed, BW, age and gender. Therefore, further studies are needed to clearly identify their impact on clinical cardiology.

CHAPTER IV- SCIENTIFIC AIMS

Small size dogs are very common in France, especially the following 7 breeds: CKCS, KCS, B, YT, D, MP, ST. These breeds are among the most common breeds seen in veterinary medical consultations, especially in urban areas. Unfortunately, these dogs are predisposed to develop DMVD, which is the most common heart disease in the canine species. Echocardiography represents the gold standard for the diagnosis of DMVD. Moreover, TTE variables, alone or in combination with cardiac and non-cardiac biomarkers assessment (plasma NT-proBNP and creatinine) can be used to assess severity of the disease and its complications, as well as to provide reliable data regarding prognosis and efficacy of medical therapy. However, other variables of interest, such as albumin, sodium, potassium chloride and PCV have been shown to be altered in the course of CHF in humans and dogs, but their prognostic significance remains unclear in dogs and warrants further investigations. To adequately interpret echocardiographic or blood variables in a dog with heart disease, they should be compared with a RI established in a healthy population. For this purpose, experts from the IFCC-CLSI (in humans medicine) and the ASVCP (in veterinary medicine) wrote specific recommendations including definition and establishment of RI. In dogs, numerous RI have been determined in veterinary medicine regarding biochemical and hematological variables for a specific breed or breed category. However, no study specifically focused on small size dogs. Regarding echocardiographic

variables as well as plasma NT-proBNP, no RI according to the IFCC-CLSI guidelines have been reported in the literature. Therefore, the purposes of the present work were to assess the effects of breed, BW, age, and gender and to determine RI according to the IFFC-CLSI guidelines for PCV, routine plasma biochemistry and plasma NT-proBNP, as well as for conventional echocardiographic and standard Doppler variables in healthy adult small size dogs.

CHAPTER V- MATERIAL AND METHODS- OVERALL PRESENTATION

The purpose of this section is to describe the material and methods used in the present work:

- In study 1: "*Basal plasma concentrations of routine variables and packed cell volume in clinically healthy adult small size dogs: effect of breed, body weight, age, and gender, and reference intervals.*"

- In study 2: "*Basal plasma concentrations of N-terminal pro-B-type natriuretic peptide in clinically healthy adult small size dogs: effect of body weight, age, gender and breed, and reference intervals.*"

- In study 3: "*Conventional echocardiographic and Doppler examinations in clinically healthy adult Cavalier King Charles Spaniels: effect of body weight, age and gender, and reference intervals.*"

I. ANIMALS

I.1. CANINE BREED

In all studies, the following small size pure-breed dogs were selected regarding to their predisposition to develop DMVD *(Chetboul et al., 2004a, Serfass et al., 2006),* as well as the breed distribution in France (*Société Centrale Canine, LOF Registration Statistics, 2012):* MP, YT, ST, D, CKCS, KCS and B.

In studies 1 and 2, all animals were breeder-owned dogs living in the area of Paris, France. The breeders were contacted by phone by the investigator before inclusion. An animal information form and an owner consent request were sent to the breeders. A maximum of ten dogs was recruited for a given breeder in order to avoid any bias on the final results due to breeder-dependent environmental effects (housing, diet, exercise, etc.). Recruitment of the different breeds was performed in a parallel design and not sequentially. In other words, the study was initiated for all the breeds simultaneously to avoid bias due to potential period effects.

In study 3, animals were breeder or client-owned dogs presented at the Cardiology Unit of Alfort, National Veterinary School of Alfort, Maisons-Alfort, France for echocardiographic screening.

In studies 1 and 2, all the 7 breeds cited above were selected (MP, YT, CKCS, KCS, D, B and ST). In study 3, only CKCS were included.

I.2. AGE

In studies 1 and 2, selected dogs were adult animals (≥ 10 months and < 9 years). For each breeder, as much as possible, dogs over a wide range of ages were included, to avoid confounding factors between age and breeder.

In study 3, the animals were breeder or client-owned adult CKCS dogs (≥ 12 months), presented for echocardiographic screening. As much as possible, dogs over a wide range of ages were included. For each study, the accurate date of birth was known and checked from the pedigree.

I.3. GENDER

A sex ratio (male:female) of 1:1 was obtained as much as possible. A sex ratio of 2:3 or 3:2 was considered as acceptable. Only intact dogs were included.

I.4. BODY WEIGHT AND BODY CONDITION SCORE

The BW had to match the standard of the breed. Additionally, in studies 1 and 2, a body condition score (BCS) was assigned for each dog using a 9-point BCS scale *(Laflamme et al., 1997).* Briefly, body condition scoring is a technique used to assess the fat (or lack thereof) found in a dog. The BCS is measured on a scale from 1 to 9 **(Figure 13)**, where 1-3 is very thin to thin, 4-5 is ideal, and 6-9 is heavy to obese.

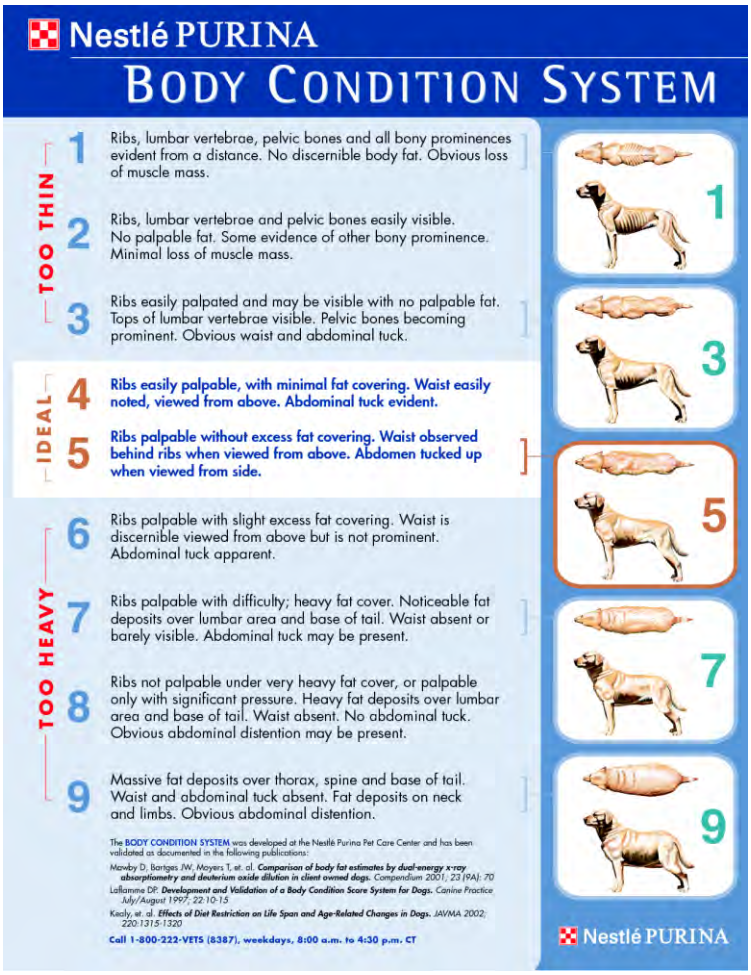

Figure 13. Body condition system in dogs based on a 9-point scale. *From Nestlé Purina.*

I.5. PHYSIOLOGICAL CONDITION

All the recruited dogs had to be healthy. Female dogs had to be non-lactating, non-pregnant and in anoestrus.

I.6. POST-PRANDIAL STATUS

Because dogs in studies 1 and 2 underwent plasma biochemistry and NT-proBNP dosages, an overnight 10-hour fasting was scheduled. Dogs could have free access to water.

II. INCLUSION CRITERIA

The inclusion criteria were as follows:

- Dogs had to be individually identified;
- Dogs had to be regularly vaccinated and dewormed (studies 1 and 2);
- Dogs had to be pure-breed dogs (*i.e.,* a copy of the pedigree had to be provided). In studies 1 and 2, a specific attention was paid to avoid inclusion of dogs from the same family (for example, siblings, parents and littermates). Alternatively, half-brothers and half-sisters could be included;
- Dogs had to be healthy; the "healthy" status was defined according to history and physical examination;
- Absence of previous history was ideal;
- Dogs had to receive a commercial pet food diet whose composition was known and documented (studies 1 and 2).

III. EXCLUSION CRITERIA

The exclusion criteria were as follows:

- Puppies and dogs under 10 months;
- Dogs aged ≥9 years (studies 1 and 2);
- Presence of a left apical systolic heart murmur consistent with DMVD;
- Body condition score superior to 7/9 or inferior to 3/9 (studies 1 and 2);
- Unfasted dogs (studies 1 and 2);
- Dogs on medication at the time of examination;
- Dogs receiving home-made diet (studies 1 and 2);
- Dogs receiving more than two treats per day (studies 1 and 2);
- The diet had been changed over the last two months before blood collection (studies 1 and 2);
- Lactating or pregnant female dogs;
- Administration of antiparasitic drugs or vaccines over the 15 days prior to blood sampling (studies 1 and 2);
- Presence of clinical signs and past history of medical events (such as infectious diseases, lameness, etc.), were not an exclusion criteria if the animal had totally recovered and if the treatment had been stopped at least three months prior to blood collection time or echocardiographic examination;
- Occurrence of clinical signs between the time of collection and two months later, assessed by phone interview of the owner. The clinical signs were considered as clinically relevant and as a factor of exclusion if they had persisted for over 72 hours and had required a therapeutic intervention (studies 1 and 2).

IV. STUDY PROCEDURES

Breeder or owner's consents were obtained for each animal before its enrolment in the study. Moreover, the latter was conducted in accordance with conditions approved by the *French Ministry of Agriculture* and all procedures were approved by a local ethic committee and in compliance with the Procedures and Principles of Good Clinical Practice *(Food and Drug Administration, Good Clinical Practice, 2001).*

IV.1. GENERAL STUDY PROCEDURES

IV.1.A. BLINDING

No blinding was requested except for the assays in studies 1 and 2 (the analyst was unaware of the dogs corresponding to the sample tubes).

IV.1.B. OWNER INTERVIEW

An information form was filled-in by the owner after she/he had given his consent. In study 1 and 2, two months after blood collection, a phone interview of the breeder was performed to assess the medical condition of the animal over the two-month period following the blood collection.

IV.1.C. IDENTIFICATION OF DOGS

Identification, breed, gender and age were checked from the pedigree and electronic microchip or tattoo. A study number was assigned for each dog.

IV.1.D. ANAMNESIS

History as well as vaccine status and deworming were documented. The common food was recorded regarding brand, manufacturer and daily amount. For bitches, the date of last oestrus was noted. Finally, absence of current medication and clinical signs over the last 4 weeks were checked.

IV.1.E. PHYSICAL EXAMINATION

In studies 1 and 2, the dog was weighed on the day of blood collection or echocardiographic examination and a BCS was attributed for each dog according to a 9-point scale (**Figure 13***, Laflamme et al., 1997).*

A complete clinical examination was performed and included rectal temperature evaluation, inspection of demeanour, hydration status, mucous color, capillary refilling time and also examination of uro-genital apparatus. Palpation included arterial femoral pulse, peripherial lymph node (mandibular, pre-scapular and popliteal) and abdomen. The respiratory rate was determined by observation of thoraco-abdominal movements and reported in respiratory cycles/min. The investigator recorded the intensity of normal sounds and the presence or the

absence of pulmonary crackles heard during the auscultation. Finally, during cardiac auscultation, the investigator explored the following areas: right and left apex and basal areas. Heart rate was determined by cardiac auscultation and reported in beat per minute.

IV.2. PROCEDURES FOR BLOOD SPECIMEN

This part concerns studies 1 and 2.

IV.2.A. BLOOD COLLECTION

To minimize the impact of circadian periodicity (especially on plasma NT-proBNP concentration), blood was collected at the same period of the day (between 10 AM and 2 PM). The dog was acclimated for a period of time (15 minutes) to the investigator and the room just before blood sampling. Blood was drawn from the left jugular vein of awake animal in the same sitting position using a 20 G needle (Neolus NN-2038S, Terumo Europe, Leuven, Belgium), a 5-mL syringe (BD Discardit II H-905S, Becton Dickinson, Fraga, Espana), a lithium heparin tube for plasma biochemistry *(Venosafe VF-054SHL, Terumo France, Guyancourt, France)* and a K3-EDTA tube *(Venosafe VF-052STK, Terumo France, Guyancourt, France)* for plasma NT-proBNP. A blood sampling form was filled in for each animal indicating information about the dog, the day and the time of blood collection, estimated duration of blood sampling, as well as potential adverse events during blood sampling. A maximum of 5 mL of blood was collected (at least 3 mL for plasma biochemistry, and 1 mL for NT-proBNP).

IV.2.B. PACKED CELL VOLUME MEASUREMENT

In study 1, well-mixed anticoagulated blood was collected into two microhematocrit tubes by capillary action. One end of each tube was sealed with a small amount of clay. The filled and sealed capillary tubes were placed into the centrifuge. Tubes were centrifuged *(Haemofuge 3522, Heraeus, MN, USA)* for five minutes at a set speed (about 12 000 g). The PCV value was read on the hematocrit reader and expressed in percent.

IV.2.C. HANDLING OF BLOOD SPECIMEN

Plasma was prepared by centrifugation for 15 minutes at *1500g (EBA 20, Hettich, Tuttlingen, Germany)*, within 30 minutes of blood sampling. For plasma biochemistry, at least 1.5 mL of the supernatant was then placed into two different Eppendorf tubes. Regarding NT-proBNP, the supernatant was placed into specific transport tubes containing protease inhibitors provided by the laboratory *(Cardiopet, IDEXX, Alfort, France).*

IV.2.D. STORAGE OF PLASMA SAMPLES

Plasma (at least 0.3 mL for NT-proBNP and 1.5 mL for plasma biochemistry) was transferred at 4°C within 45 minutes of blood sampling and then stored at -80°C within 6 hours.

IV.2.E. SHIPMENT OF PLASMA SAMPLES

Plasma samples (1.5 mL for biochemistry and 0.5 for NT-proBNP) were sent once a week to the laboratory (at 4 °C for biochemistry and -80°C for NT-proBNP).

IV.3. ANALYTICAL METHOD

IV.3.A. PLASMA BIOCHEMISTRY

In study 1, creatinine, urea, phosphate, sodium, potassium, chloride, total proteins and calcium, albumin, glucose, ALP, ALT, AST, triglycerides and cholesterol were assessed in the same laboratory *(Laboratoire Vebiotel, Arcueil, France).* Electrolytes and substrates/enzymes were determined by direct potentiometry with specific electrodes, absorption spectrophotometry and colorimetric methods, respectively *(Konelab 60, ThermoClinical - 95, Cergy Pontoise, France).* Method of analysis for plasma analytes measured in this study and results of the between-run coefficient of variations for control solutions are provided in **Tables 15 and 16**.

Table 15. Method of analysis for plasma urea, creatinine, total proteins, albumin and liver enzymes measured in a first study and results of the between-run coefficient of variations for control solutions *(1- Abtrol, Thermo Electron Co, Madison, WI, USA; 2- Nortrol, Thermo Electron Co, Madison, WI, USA).*

Analyte	Methodology	Control concentration 1	Between-run coefficient of variation (%)	Control concentration 2	Between-run coefficient of variation (%)
Urea (mmol/L)	Colorimetric (urease and glutamate dehydrogenase)	14.8	3.90	6.5	3.22
Creatinine (µmol/L)	Colorimetric (modified Jaffe: kinetic measurement)	337	4.49	156	4.37
Total proteins (g/L)	Colorimetric (Biuret method)	45.8	2.73	63.1	2.41
Albumin (g/L)	Colorimetric (bromocresol green)	28.6	2.16	41.3	1.97
ALT (U/L)	Absorption spectrophotometry (alanine/oxoglutarate) without pyridoxal phosphate (30°C)	157	3.13	40	5.07
AST (U/L)	Absorption spectrophotometry (L-aspartate/glutarate) without pyridoxal phosphate (30°C)	158	2.46	36	2.30
ALP (U/L)	Absorption spectrophotometry (P nitro phenylphosphate) (30°C)	275	5.10	80	6.90

Table 16. Method of analysis for plasma glucose, electrolytes, phosphate, cholesterol and triglycerides measured in a first study and results of the between-run coefficient of variations for control solutions *(1- Abtrol, Thermo Electron Co, Madison, WI, USA; 2- Nortrol, Thermo Electron Co, Madison, WI, USA).*

Analyte	Methodology	Control concentration 1	Between-run coefficient of variation (%)	Control concentration 2	Between-run coefficient of variation (%)
Glucose (mmol/L)	Colorimetric (glucose oxidase/peroxidase)	15.9	1.92	5.0	2.05
Sodium (mmol/L)	Potentiometric (ion selective electrode)	152	1.38	137	1.10
Potassium (mmol/L)	Potentiometric (ion selective electrode)	6.5	1.92	4.2	1.85
Chloride (mmol/L)	Potentiometric (ion selective electrode)	117	1.82	100	2.02
Calcium (mmol/L)	Colorimetric (arsenazo III)	3.18	2.81	2.25	2.15
Phosphate (mmol/L)	Colorimetric (molybdate ammonium)	2.04	4.16	1.03	5.87
Cholesterol (mmol/L)	Colorimetric (cholesterol esterase)	6.79	1.83	4.28	2.36
Triglycerides (mmol/L)	Colorimetric (lipase/peroxydase)	2.09	3.20	0.75	3.30

IV.3.B. PLASMA N-TERMINAL PRO-BRAIN NATRIURETIC PEPTIDE

Plasma NT-proBNP assays (study 2) were performed in the same laboratory *(Laboratoire Idexx, Alfort, France).* Plasma NT-proBNP concentration was measured using EDTA-potassium samples and a commercially available canine specific assay *(Cardiopet, IDEXX, Alfort, France).* This sandwich ELISA assay has already been used and validated for diagnostic purposes in the dog *(Boswood et al., 2008; Zieba et al., 2008).* The inter- and intra-assay coefficient variations provided by the laboratory were between 5.3 and 9.2% and between 2.1 and 10.7% (for concentrations ranging from 900 to 2400 pmol/L), respectively.

IV.4. CARDIOVASCULAR EXAMINATION

A complete cardiovascular examination, including conventional echocardiography and standard Doppler examination, as well as systemic arterial blood pressure measurement was scheduled for outlier dogs in study 2 (see Chapter VI: Results and Original Articles). In study 3, all dogs underwent conventional echocardiography and standard Doppler examination.

IV.4.A. CONVENTIONAL ECHOCARDIOGRAPHY AND STANDARD DOPPLER EXAMINATION

Conventional echocardiography and standard Doppler examinations were performed by trained observers in awake dogs gently restrained in standing position, using continuous ECG monitoring

with an ultrasound unit *(Vingmed system 5, Vivid 5, Vivid 7 dimension and Vivid 7 BT03, Vivid I BT 10 SW appl. R 10.3.0 General Electric Medical System, Waukesha, Wis)* equipped with 3S (1.5-3.5 MHz), 5S (2.2-5 MHz), and 7S (3.5-8 MHz) phased-array transducers, as previously described *(Chetboul et al., 2004b).*

Measurements of the Ao and LA diameters **(Figure 14)** were obtained at end-diastole by a bidimensional method using the right parasternal transaortic short-axis view using the calipers position described by *Hansson et al. (2002)*, and the LA/Ao ratio was then calculated *(Chetboul et al., 2005).*

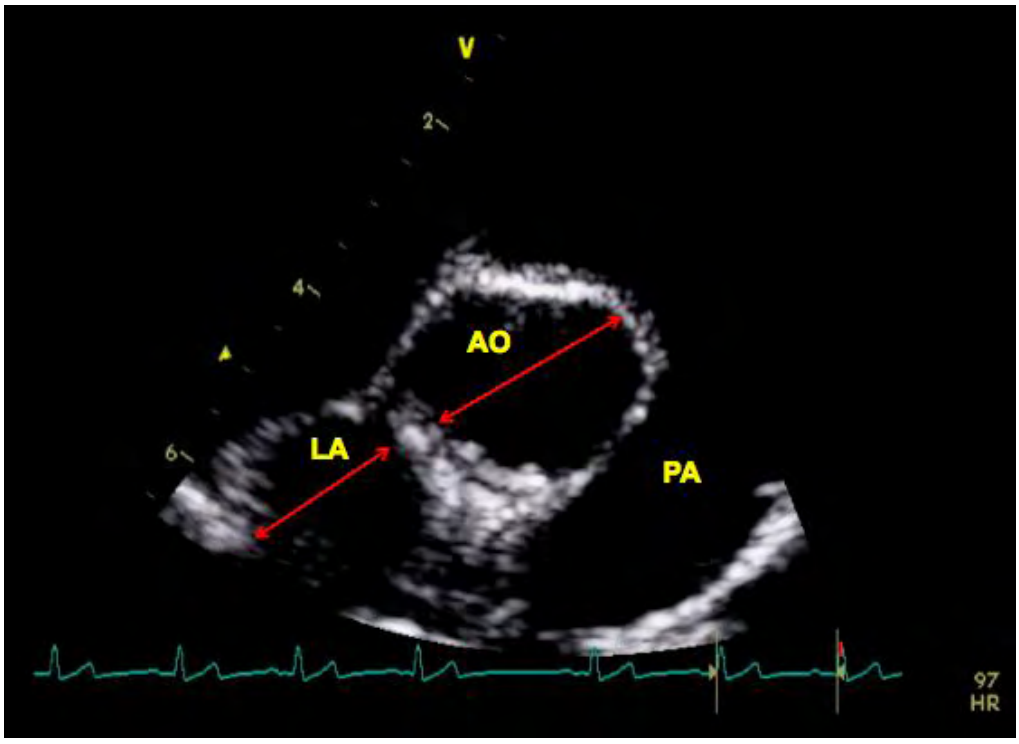

Figure 14. Right parasternal transaortic short-axis view in bidimensional mode showing positioning of calipers (double arrows) allowing calculation of the left atrium (LA) on aorta (AO) ratio. *HR, heart rate (in beat per minute); PA, pulmonary artery. Photograph: Cardiology Unit of Alfort.*

Ventricular measurements were obtained according to the recommendations of the American Society of Echocardiography *(Sahn et al., 1978)* from the right parasternal location (transventricular short-axis view), using the bidimensional guided M-mode echocardiography **(Figure 15)**. Measurements were made of the LVED and LVES, left ventricular free wall and interventricular septum thicknesses in diastole and in systole. The left ventricular fractional shortening (expressed in %) was then calculated using the following formula: (LVED-LVES)/LVED.

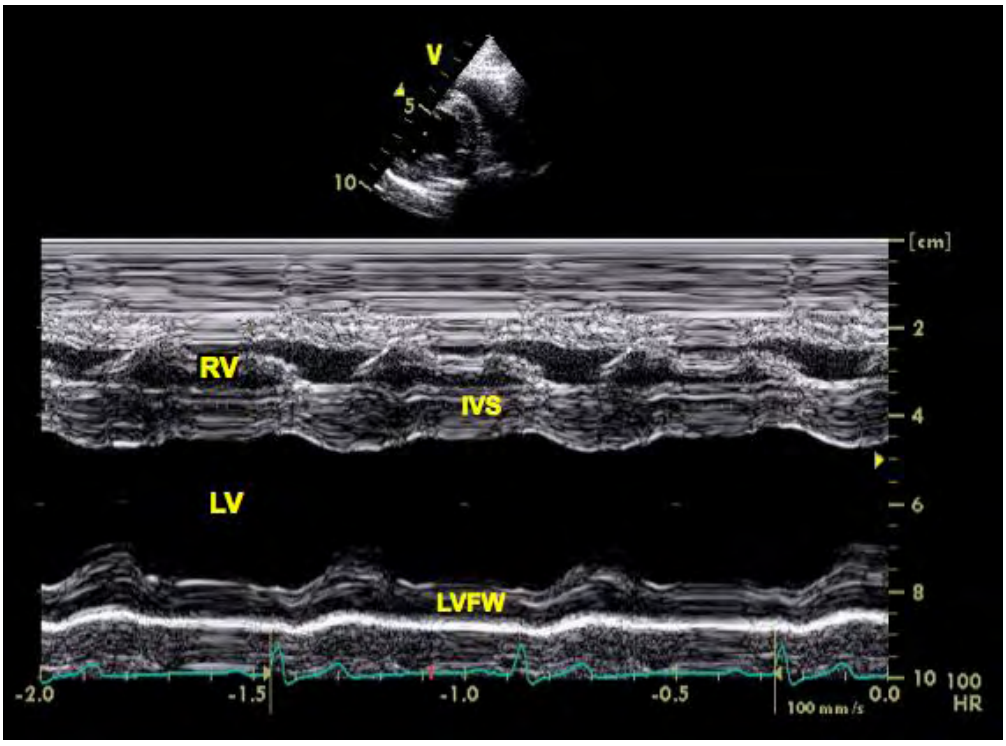

Figure 15. M-mode right parasternal left ventricular short-axis view. *HR, heart rate (in beats per minute); IVS: interventricular septum; LV, left ventricle; LVFW, left ventricular free wall; RV, right ventricle. Photograph: Cardiology Unit of Alfort.*

The maximal systolic pulmonary and aortic flow velocities were measured using the right parasternal transaortic short-axis and the left apical 5-chamber views, respectively **(Figure 16)**. Additionally, maximal early (E) and late (A) diastolic mitral flow velocities were determined by pulsed-wave Doppler using the left apical 4-chamber view.

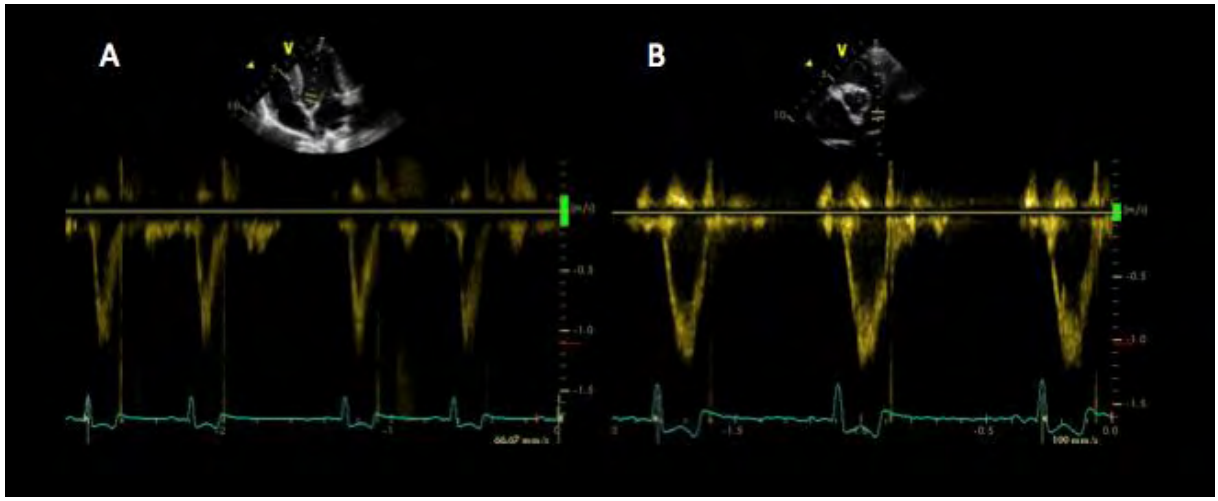

Figure 16. Pulsed wave Doppler mode showing the maximal systolic aortic velocity, on the left apical long axis 4-chamber view (A) and the maximal systolic pulmonary velocity (B), on the right parasternal transaortic short-axis view. *Photograph: Cardiology Unit of Alfort.*

In study 2, conventional echocardiographic and Doppler variables of outlier dogs were compared with the previously established reference ranges *(Gonçalves et al., 2002; Chetboul et al., 2005).* These reference ranges are provided in **Tables 17 and 18**.

Table 17. Reference ranges proposed for the conventional and pulsed wave Doppler modes variables assessed in the present study. *From Chetboul et al. Use of quantitative two-dimensional color tissue Doppler imaging for assessment of left ventricular radial and longitudinal myocardial velocities in dogs. Am J Vet Res 2005;66:953-961.*

Variable	Reference range	Variable	Reference range
Fractional shortening (%)	30.1-40.0	Peak early (E) mitral diastolic velocity (m/s)	0.58-1.17
Left atrium to aorta ratio	0.52-1.13	Peak late (A) mitral diastolic velocity (m/s)	0.39-0.86
Peak aortic velocity (m/s)	0.92-1.88	Mitral E/A ratio	0.92-2.72
Peak pulmonary velocity (m/s)	0.50-1.50		

Table 18. Equation used to predict the reference ranges for the 6 body-weight dependent M-mode variables assessed in the present study (i.e., end-diastolic and end-systolic left ventricular wall thicknesses and internal diameters). *From Gonçalves et al. Linear, logarithmic, and polynomial models of M-mode echocardiographic measurements in dogs. Am J Vet Res 2002;63:994-999.*

M-mode measurement	Mathematical model	Equation for 95% prediction interval
Interventricular septum during diastole	Simple linear	$y = 8.83 + 0.105x \pm 2(1.59\sqrt{1.015 + [(x - 27.4)^2/25,947.2]})$
Left ventricular dimension during diastole	Second-order polynomial	$y = 19.4 + 0.769x - 0.005x^2 \pm 2(3.05\sqrt{1.015 + [(x - 27.4)^2/25,947.2]})$
	Logarithmic	$y = 5.66 + 9.416\ln(x) \pm 2(2.9\sqrt{1.015 + [(x - 27.4)^2/25,947.2]})$
Left ventricular wall during diastole	Simple linear	$y = 7.09 + 0.08x \pm 2(1.55\sqrt{1.015 + [(x - 27.4)^2/25,947.2]})$
Interventricular septum during systole	Simple linear	$y = 11.75 + 0.146x \pm 2(1.83\sqrt{1.015 + [(x - 27.4)^2/25,947.2]})$
Left ventricular dimension during systole	Second-order polynomial	$y = 10.68 + 0.568x - 0.004x^2 \pm 2(2.54\sqrt{1.015 + [(x - 27.4)^2/25,947.2]})$
	Logarithmic	$y = 1.59 + 6.525\ln(x) \pm 2(2.53\sqrt{1.015 + [(x - 27.4)^2/25,947.2]})$
Left ventricular wall during systole	Simple linear	$y = 10.35 + 0.136x \pm 2(2.32\sqrt{1.015 + [(x - 27.4)^2/25,947.2]})$

Moreover, in study 2, the left apical 4-chamber view was used for color-flow Doppler examination of the tricuspid valve. When tricuspid regurgitation (TR) was identified, the peak systolic TR velocity was quantitatively assessed using continuous-wave Doppler mode, and the systolic pulmonary arterial pressure was calculated by applying Bernoulli's equation and adding the estimated right atrial pressure to the systolic right ventricle-to-right atrium pressure gradient, as previously described *(Serres et al., 2006)*. Similarly, all ultrasound examinations included a color Doppler analysis of the pulmonary valve flow to detect a potential diastolic pulmonary regurgitant jet. When such a regurgitant jet was identified, the maximal regurgitant flow velocity was assessed using continuous Doppler mode, and high velocity values (i.e., ≥2.0 m/s) were considered for the diagnosis of diastolic pulmonary arterial hypertension *(Serres et al., 2006)*. Finally, in studies 2 and 3, the morphological aspect of the mitral valve apparatus (i.e., mitral leaflets and *chordae tendineae*) was also assessed, and a color-flow Doppler examination was performed using right and left apical 4-chamber views in order to exclude DMVD (thickening of mitral valve leaflets and *chordae tendineae* and/or mitral valve prolapse associated with mitral

regurgitation). Heart rate was calculated by ECG monitoring during each echocardiographic examination via an ECG taken during the same cardiac cycles used for the M-mode measurements.

IV.4.B. SYTEMIC ARTERIAL BLOOD PRESSURE MEASUREMENT

Systemic systolic arterial blood pressure was indirectly measured before each echocardiographic examination in the conscious outlier dogs (study 2), in accordance with the ACVIM consensus statement *(Brown et al., 2007),* by the same trained observer using the Doppler method *(811-BL, Parks Medical Electronics Inc. Aloha, Ore, USA;* **Figure 17***)*. Dogs were gently held by the owner in sternal recumbency. An inflatable cuff *(Soft-cuf, Ref 2422, 2 cm large, Parks Medica, USA)* was placed on the tail, as previously described *(Chetboul et al., 2010).* A period of acclimatization was allowed for each patient before blood pressure was measured. Several measurements were performed over 5-10 minutes to obtain a stable set of 5 values and the mean was used for statistical analyses. Systemic arterial hypertension and hypotension were defined as systolic arterial blood pressure values >160 mmHg in unstressed animals and <100 mmHg, respectively *(Brown et al., 2007).*

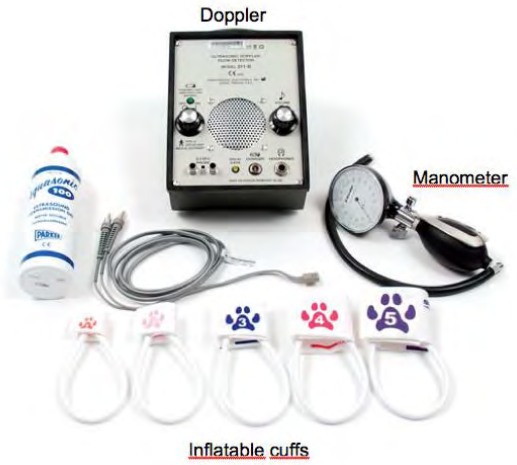

Figure 17. Material used for systemic arterial blood pressure measurement using the Doppler method. *Source: www.dispomed.com*

IV.5. STATISTICAL ANALYSIS

IV.5.A. DESCRIPTIVE STATISTICS

For each tested variable, median, range or interquartile ranges were calculated using a specific software *(Systat version 8.0, SPSS Inc., Chicago, IL, USA).*

IV.5.B. GENERAL LINEAR MODEL

Effects of breed, age, gender and BW on plasma biochemistry and NT-proBNP concentrations (studies 1 and 2) and effect of age, gender and BW on echocardiographic variables (study 3) were

tested using a statistical software package *(Systat version 8.0, SPSS Inc, Chicago, IL, USA).* A value of $P<0.05$ was considered significant. Age and BW in each breed were compared by ANOVA (studies 1 and 2).

In studies 1 and 2, effects of breed, sex, age, and BW on the variables were tested using the following linear mixed effects model:

$$\mathbf{Y=\mu+Breed+Sex+aAge+bBW+ (Breed\ x\ Age) + (Breed\ x\ BW) + (Breed\ x\ Sex) + \varepsilon}$$

Where Y is the value of the plasma variable; μ is a constant term; a and b are the slope coefficients for age and BW irrespective of the breed. The other terms denote interactions between breed and age, breed and body weight, and breed and sex. ε is the residual term of the model.

In study 3, effects of sex, age, and BW on the variables were tested using the following linear mixed effects model:

$$\mathbf{Y=\mu+Sex+aAge+bBW+\varepsilon}$$

Where Y is the value of the echocardiographic variable; μ is a constant term; Age and BW are continuous variables, a and b are the slope coefficients for Age and BW, and ε is the residual term of the model.

IV.5.C. DETERMINATION OF REFERENCE INTERVAL

Identification of outliers and determination of RI were performed according to the current CLSI guidelines *(CLSI, 2008)*. Native and Box-Cox transformed data were first tested for normality by use of the Anderson-Darling test. The set data were then examined for outliers. Tukey criterion was applied when the distribution was Gaussian. When the data distribution remained non-Gaussian after Box-Cox transformation, the Tukey method was not applicable and visual inspection of values in both tails of the distribution was used. When a value was deemed questionable, the following criteria were used to decide whether to remove a dog from the study: any abnormality concerning medical history or clinical examination and an atypical value for more than one analyte from the same dog (study 1) or any abnormality concerning medical history, clinical and echo-Doppler examinations as well as systemic arterial blood pressure, and plasma blood urea nitrogen (BUN) and creatinine (study 2) or any abnormality concerning clinical and echo-Doppler examinations (study 3). Reference intervals were defined as central 95% intervals bounded by the 2.5th and 97.5th percentiles. Upper and lower limits of the reference interval with the 90% CI were determined in the global population by a nonparametric approach, using a specific software *(Geffré et al., 2011).*

CHAPTER VI- RESULTS

This part summarizes results of the 3 studies performed in the present work. The corresponding original articles that have been published are presented in the appendix section.

- Article 1 (corresponding to study 1): "*Basal plasma concentrations of routine variables and packed cell volume in clinically healthy adult small size dogs: effect of breed, body weight, age, and gender, and establishment of reference intervals"* by Misbach C, Chetboul V, Concordet D, Gruet P, Speranza C, Hoffmann AC, Rocha A, Balouka D, Petit AM, Trehiou-Sechi E, Pouchelon JL and Lefebvre HP. Published in Veterinary Clinical Pathology in September 2014 (volume 43, pages 371 to 380).

- Article 2 (corresponding to study 2): "*Basal plasma concentrations of N-terminal pro-B-type natriuretic peptide in clinically healthy adult small size dogs: effect of breed, body weight, age, and gender, and establishment of reference intervals"* by Misbach C, Chetboul V, Concordet D, Gruet P, Speranza C, Hoffmann AC, Rocha A, Balouka D, Petit AM, Trehiou-Sechi E, Pouchelon JL and Lefebvre HP. Published in Research in Veterinary Science in December 2013 (volume 95, pages 879 to 885).

- Article 3 (corresponding to study 3) on "*Conventional and Doppler echocardiography in clinically healthy adult Cavalier King Charles Spaniels: effect of body weight, age, and gender, and establishment of reference intervals"* by Misbach C, Lefebvre HP, Concordet D, Gouni V, Trehiou-Sechi E, Petit AM, Damoiseaux C, Leverrier A, Pouchelon JL and Chetboul V. Published in the Journal of Veterinary Cardiology in June 2014 (volume 16, pages 91 to 100).

I. ARTICLE 1: Basal plasma concentrations of routine variables and packed cell volume in clinically healthy adult small size dogs: effect of breed, body weight, age, and gender, and reference intervals.

Basal plasma concentrations of routine variables and packed cell volume in clinically healthy adult small size dogs: effect of breed, body weight, age, and gender, and establishment of reference intervals by Misbach C, Chetboul V, Concordet D, Gruet P, Speranza C, Hoffmann AC, Rocha A, Balouka D, Petit AM, Trehiou-Sechi E, Pouchelon JL and Lefebvre HP. Published in Veterinary Clinical Pathology in September 2014 (volume 43, pages 371 to 380).

Objectives

The primary objective of this prospective study was to assess the potential effect of breed on routine plasma analytes in dogs from 7 different small size breeds and to assess the effects of

BW, age, and gender on the same plasma variables. A secondary objective was to use this small size dog population as a reference sample group to determine RI according to the procedures recommended by the CLSI guidelines.

Material and Methods

For this purpose, healthy small size dogs from 7 breeds (B, CKCS, KCS, MP, ST, YT and D) were prospectively included in this study. Animals were assessed as healthy on the basis of a complete physical examination and history. Blood was sampled in standardized conditions. Packed cell volume was measured immediately after sampling. Assays for 15 routine plasma variables (urea, creatinine, total proteins, albumin, glucose, ALT, AST, ALP, sodium, potassium, chloride, calcium, phosphate, cholesterol, and triglycerides) were performed by the same commercial laboratory using direct potentiometry with specific electrodes, and absorption spectrophotometry and colorimetric methods for electrolytes and substrates/enzymes, respectively. Effect of breed, BW, age and gender was tested using a general linear model. Identification of outliers and determination of RI were performed according to the current the CLSI guidelines.

Results

One hundred and fifty four healthy sexually intact small size dogs from 7 breeds were included in the study. Although a statistically significant effect ($P<0.05$) of breed, BW, gender, and age was evidenced for respectively 7/16, 8/16, 4/16, and 3/16 tested variables, establishment of breed, gender-, age- or BW-specific RI for most of the variables was considered clinically irrelevant.

More interestingly, according to the analyte, 3.8% to 76.6% of the observed values were lower than the lower limit of the laboratory's RI used routinely for 9/12 tested variables and 4.5% to 9.7% of the observed values were higher than the upper limit of the laboratory's RI for 7/12 tested variables, thus indicating that the laboratory's RI was not appropriate for all variables except plasma glucose and chloride **(Table 19)**.

Conclusion

This study seems to indicate that the determination of specific RI for routine variables in small size dogs could be clinically relevant. Inversely, the present results showed that the decision to partition RI into subclasses for these 16 routine variables in the studied population according to criteria such as breed, BW, age and gender seemed not to be of clinical interest.

Table 19. Reference intervals established from the 154 reference individuals and comparison with the laboratory's reference intervals. *BCG, Gaussian after Box-Cox transformation; CI, confidence interval; LL, lower limit; NG, non-Gaussian; UL, upper limit.*

Analyte	Distribution	LL (90% CI)	UL (90% CI)	LL-UL of the laboratory	Number (%) of values < LL of the lab	Number of values >LL of the lab
Packed cell volume (%)	BCG	35.9 (34-37)	56 (54-57)	37-55	6 (3.8%)	3 (1.9%)
Urea (mmol/L)	NG	3.3 (3.3-3.3)	11.6 (8.3-13.3)	3.3-8.3	0	7 (4.5%)
Creatinine (µmol/L)	NG	45 (45-54)	90 (81-99)	54-144	44 (28.6%)	0
Total proteins (g/L)	NG	47.6 (43-49)	67.1 (65-70)	60-80	118 (76.6%)	0
Albumin (g/L)	NG	23.9 (22-24)	33.0 (32-34)	30-40	118 (76.6%)	0
Glucose (mmol/L)	NG	3.9 (3.4-3.9)	6.2 (6.2-7.3)	3.9-6.2	2 (1.3%)	2 (1.3%)
ALT (U/L)	NG	19 (4-22)	261 (133-296)	<80	-	15 (9.7%)
AST (U/L)	NG	3 (2-10)	66 (54-129)	10-50	6 (3.9%)	9 (5.8%)
ALP (U/L)	NG	7 (1-10)	92 (76-113)	<80	-	7 (4.5%)

Analyte	Distribution	LL (90% CI)	UL (90% CI)	LL-UL of the laboratory	Number (%) of values < LL of the lab	Number of values >LL of the lab
Sodium (mmol/L)	NG	137 (132-139)	152 (152-154)	145-160	69 (44.8%)	0
Potassium (mmol/L)	NG	3.5 (3.3-3.5)	4.6 (4.4-5.2)	3.6-5.8	11 (7.1%)	0
Chloride (mmol/L)	NG	106 (102-107)	115 (115-119)	95-115	0	1 (0.6%)
Calcium (mmol/L)	NG	2.12 (2.00-2.20)	2.67 (2.62-3.29)	2.25-2.99	9 (5.8%)	1 (0.6%)
Phosphate (mmol/L)	NG	0.74 (0.55-0.81)	2.65 (1.84-2.91)	0.81-1.61	6 (3.9%)	13 (8.4%)
Cholesterol (mmol/L)	NG	1.81 (1.29-2.32)	7.48 (6.71-8.26)	0.52-6.45	0	8 (5.2%)
Triglycerides (mmol/L)	NG	0.23 (0.23-0.34)	2.51 (1.60-5.24)	0.23-1.71	0	6 (3.8%)

II. ARTICLE 2: Basal plasma concentrations of N-terminal pro-B-type natriuretic peptide in clinically healthy adult small size dogs: effect of body weight, age, gender and breed, and reference intervals

Basal plasma concentrations of N-terminal pro-B-type natriuretic peptide in clinically healthy adult small size dogs: effect of body weight, age, gender and breed, and reference intervals by Misbach C, Chetboul V, Concordet D, Gruet P, Speranza C, Hoffmann AC, Rocha A, Balouka D, Petit AM, Trehiou-Sechi E, Pouchelon JL and Lefebvre HP. Published in Research in Veterinary Science in December 2013 (Volume 95, pages 879 to 885).

Objectives

The aims of this prospective study were 1) to evaluate plasma concentrations of NT-proBNP, 2) to identify potential effects of breed, BW, age, and gender and 3) to establish RI according to the statistical procedures recommended by the CLSI guidelines, in a large population of healthy adult small size dogs from different breeds known to be predisposed to DMVD.

Material and Methods

For this purpose, healthy small size dogs from 7 breeds (B, CKCS, KCS, MP, ST, YT and D) were prospectively included in this study. Animals were assessed as healthy on the basis of a complete physical examination, history and routine plasma panel. Blood was sampled in standardized conditions and assays for plasma NT-proBNP was performed by an ELISA method.

Effect of breed, BW, age and gender was tested using a general linear model. Identification of outliers and determination of RI were performed according to the current CLSI guidelines.

Results

The reference sample group included 154 sexually intact healthy small size dogs from 7 breeds. The commercial laboratory did not quantify the NT-proBNP concentration when it exceeded 2842 pmol/L. Nine out of the 154 tested dogs had plasma NT-proBNP >2842 pmol/L, therefore their NT-proBNP value was set at 2842 pmol/L for statistical analysis. Despite standardized procedures with strict inclusion and exclusion criteria, plasma NT-proBNP showed a high individuality **(Figure 18).**

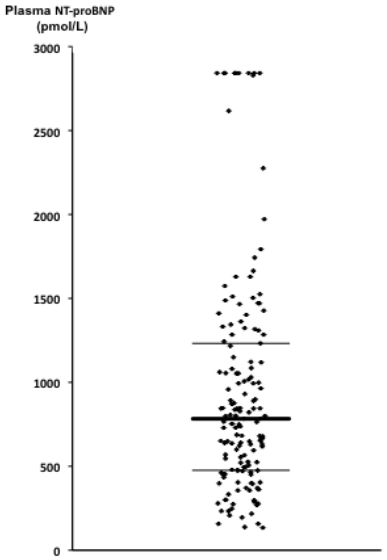

Figure 18. Distribution of plasma N-terminal pro-brain-type natriuretic peptide (NT-proBNP) concentrations among the 154 healthy small size dog population. *The thick horizontal bar represents the median (i.e., 783 pmol/L) and the thin horizontal bars represent the first and third quartiles (i.e., 476 and 1232 pmol/L, respectively).*

The inter-individual CV in the general population was 96% and the within-breed inter-individual CV ranged from 62% (CKCS) to 100% (MP). Although no age or breed or BW effect was observed on plasma NT-proBNP, a significant sex effect (P=0.01) was shown (males: 683 pmol/L [404-892]; females: 844 pmol/L [550-1327]). Moreover, 11/154 dogs (7.1%) had plasma NT-proBNP >2617 pmol/L, and were statistically considered as outliers. These dogs underwent a complete cardiovascular work-up, including arterial blood pressure measurement, conventional echocardiography and Doppler examinations. Plasma BUN and creatinine as well as re-assessment of plasma NT-proBNP were also scheduled 3 months after the first sampling. All dogs had normal cardiovascular examination, normal blood pressure and normal plasma BUN and creatinine concentrations. At recheck, only 4/11 dogs had plasma NT-proBNP values that still exceeded the upper limit of quantification of the assay **(Figure 19)** and the 7 remaining dogs showed a 1.2 to 6-fold decrease from their initial NT-proBNP value. The proposed RI for plasma NT-proBNP was 157-2842 pmol/L (i.e., the upper limit of the RI corresponded to the limit of quantification).

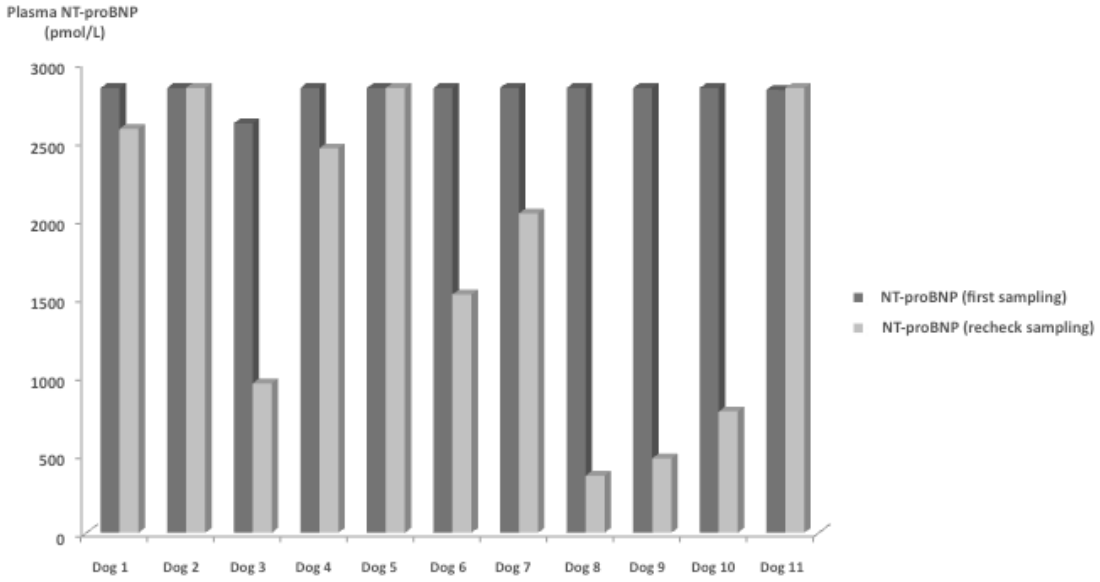

Figure 19. Comparison between plasma N-terminal pro-brain-type natriuretic peptide (NT-proBNP) concentrations assessed in the 11 outlier dogs at first sampling and 3 months later.

Conclusion

This study suggests that plasma NT-proBNP concentration is characterized by a high inter-individual variability in healthy adult small size dogs. Therefore, a single plasma value in a healthy small size dog should be interpreted with caution. Moreover, a gender effect has been demonstrated, with females having higher concentrations than males, but the clinical relevance of partitioning the RI according to gender needs further investigations.

III. ARTICLE 3: Echocardiography and conventional Doppler examination in clinically healthy adult Cavalier King Charles Spaniels: effect of breed, body weight, age, and gender, and establishment of reference intervals

Echocardiography and conventional Doppler examination in clinically healthy adult Cavalier King Charles Spaniels: effect of breed, body weight, age, and gender, and establishment of reference intervals by Misbach C, Lefebvre HP, Concordet D, Gouni V, Trehiou-Sechi E, Petit AM, Damoiseaux C, Leverrier A, Pouchelon JL and Chetboul V. Published in the Journal of Veterinary Cardiology in June 2014 (volume 16, pages 91 to 100).

Objectives

The aims of this prospective third study were 1) to assess the potential effect of BW, age, and gender on the most common standard echocardiographic and conventional Doppler variables in a large population of clinically healthy adult CKCS, and 2) to establish the corresponding RI according to the statistical procedure recommended by the CLSI guidelines.

Material and Methods

The reference sample group consisted of breeder- and client-owned healthy adult (≥12 months) CKCS presented for cardiac screening at the Cardiology Unit of Alfort (National Veterinary School of Alfort, France) between March 2001 and January 2013. Animals were considered healthy on the basis of a complete physical examination. Standard echocardiography and conventional Doppler examinations were performed by trained observers, with at least 3 years of experience in cardiology, in awake dogs gently restrained in standing position, using continuous electrocardiogram monitoring with an ultrasound unit equipped with phased-array transducers, as previously described and validated. Effect of BW, age and gender was tested using a general linear model. Identification of outliers and determination of RI were performed according to the current CLSI guidelines.

Results

A total of 134 healthy adult CKCS were included. A significant BW effect was observed for all variables except the M-mode, 2D and Doppler ratios, i.e., fractional shortening (FS%), LA/Ao ratio, and the E/A ratio, the two latter being the sole variables not significantly affected by any of the tested covariates. Unlike these ratios, all imaging variables (n=12) were significantly affected by BW as well as gender (4/12) and/or age (4/12). The clinical relevance of these results was discussed for each variable with regard to the observers' variability. Only the BW effect on M-mode variables was considered as clinically relevant and regression-based RI were built

accordingly **(Table 20)**. Moreover, although all the M-mode echocardiographic RI established in the present study were within the predictive RI calculated with Cornell's formula (which is an allometric scaling used to predict percentiles according to BW and developed from a large population of canine small, large and giant breeds), all were smaller than this latter **(Table 21)**.

Table 20. Predicted regression-based reference intervals (lower and upper limits) according to body weight for the six M-mode variables with a significant body weight effect assessed in a population of healthy adult Cavalier King Charles Spaniels (n=134). *LVDd and LVDs, end-diastolic and end-systolic left ventricular diameters, respectively; LVFWd and LVFWs, end-diastolic and end-systolic left ventricular free wall thicknesses, respectively; IVSd and IVSs, end-diastolic and end-systolic interventricular septum thicknesses, respectively.*

Body weight (kg)	LVDd (mm)	LVDs (mm)	LVFWd (mm)	LVFWs (mm)	IVSd (mm)	IVSs (mm)
5	20.2-29.8	10.6-20.3	4.0-7.0	6.6-11.3	4.3-7.7	6.0-11.6
6	21.3-30.9	11.2-20.8	4.2-7.3	7.1-11.7	4.5-7.8	6.4-11.9
7	22.5-31.9	11.8-21.3	4.5-7.5	7.6-12.2	4.6-7.9	6.7-12.2
8	23.6-33.0	12.3-21.9	4.7-7.7	8.1-12.6	4.8-8.1	7.1-12.5
9	24.6-34.1	12.9-22.4	4.9-7.9	8.5-13.1	4.9-8.2	7.4-12.9
10	25.8-35.2	13.5-23.0	5.2-8.1	9.0-13.5	5.1-8.3	7.7-13.2
11	26.8-36.3	14.0-23.6	5.4-8.4	9.4-14.0	5.2-8.5	8.0-13.5
12	27.8-37.4	14.5-24.1	5.6-8.6	9.9-14.5	5.3-8.6	8.3-13.9
13	28.8-38.5	15.0-24.7	5.8-8.8	10.3-14.9	5.4-8.8	8.6-14.2

Table 21. M-mode echocardiographic reference intervals (2.5th and 97.5th percentiles) from 134 healthy adult Cavalier King Charles Spaniels determined according to the Clinical and Laboratory Standards Institute *(CLSI, 2008)* statistical procedures (A), and predictive reference intervals calculated in the same population using Cornell's formula (*Cornell et al., 2004;* B). *See Table 20 for remainder of key.*

M-mode echocardiographic variable	A	B
LVDd (mm)	23.4-35.6	21.5-40.6
LVDs (mm)	12.9-23.6	12.0-27.7
LVFWd (mm)	5.0-8.3	4.9-13.2
LVFWs (mm)	8.2-13.8	8.1-19.1
IVSd (mm)	5.1-8.3	4.9-13.0
IVSs (mm)	7.6-13.0	7.3-17.3

Conclusion

In conclusion, although predictive RI assessed according to Cornell's formula seem to be acceptable for CKCS dogs, the present results suggest that determining specific RI for echocardiographic and Doppler variables in this breed is relevant. The present report also demonstrates a statistically significant and clinically relevant BW effect on M-mode echocardiographic variables including end-diastolic and end-systolic left ventricular wall thicknesses and diameters. The BW should therefore be considered when interpreting echocardiographic values in CKCS, except for the 2D, M-mode and Doppler ratios, i.e., LA/Ao, FS% and mitral E/A, respectively.

CHAPTER VII- DISCUSSION, CONCLUSION AND PERSPECTIVES

Aims of the study

The main goal of this work was to apply the concept of RI in veterinary cardiology through various clinical settings. One specific objective included determination of RI within a population of small size dogs for 1) PCV and routine plasma biochemisty variables, 2) the plasma biomarker NT-proBNP, and 3) standard echocardiographic and conventional Doppler variables. For such a purpose, the procedures recommended by the IFCC-CLSI guidelines *(Solberg, 1987a, 1987b, 1988; Solberg and Stamm, 1991; Petit Clerc and Solberg, 1987; Dybkaér 1987; CLSI, 2008)* were applied. Although these guidelines have been generated specifically for clinical laboratory, we chose to extend the statistical recommendations to determine RI for echocardiographic variables in the CKCS breed. Another specific objective of this work was to assess the effect of covariates (breed, BW, age and gender) on the tested blood or imaging variables, by using a general linear model. Some of these effects have already been identified to different degrees in the literature, as previously stated in Chapter III.

Selection of the reference sample group

In the present work, sexually intact purebred dogs corresponding to the standard provided by the *French kennel Club (Société Centrale Canine)* were chosen to represent the reference population.

Only small size breeds were recruited because they are very popular in France. Hence, according to the figures provided by the *French Kennel Club*, 4 out of the 7 small breeds studied here (YT, ST, MP, CKCS, KC, B and D) are among the 20 breeds with the highest number of registrations to *"Le Livre des Origines Françaises (LOF)"*. Besides, within the 207 987 registrations to LOF in 2012, the CKCS, YT, ST and D were ranked in position 4, 9, 16 and 20, respectively. Moreover, these small size breeds account for an important part of dogs examined in cardiology consultations due to their predisposition to develop DMVD, which is the most common heart disease in dogs *(Buchanan, 1977; Thrusfield et al., 1985).* A French study performed in the area of Paris, France *(Serfass et al., 2006)* and including 942 small size breeds (i.e., YT, MP, B, D, ST) with a mean age of 6.5 years, showed that the prevalence of DMVD (confirmed by the presence of left apical systolic heart murmur at cardiac auscultation) ranged from 8.5 % (YT) to 22.7% (MP) depending on the breed. Moreover, another French study *(Chetboul et al., 2004a)* conducted in the same geographic location and including a large number of dogs (n=451) from the same breed (CKCS) demonstrated a high prevalence of DMVD (i.e., 40.6%) in relatively young dogs (mean age of 4.5 years). Another breed predisposed to DMVD in France is the Lhassa Apso, with a prevalence of affected dogs that reached 6.5% in one previous study (*Serfass et al., 2006).* This breed was not recruited in the present study for technical reasons, because a too small number of breeders was found in the area of Paris, representing thus a limitation of the study.

Regarding age, only adult dogs (i.e., > 10 months) were included in the study, because DMVD is an acquired heart disease. This observation was confirmed in one previous study, where the age at which 20% of a small breed developed a murmur secondary to DMVD was 8.6, 9.5, 10.4, 11.1 and 12.7 years in respectively ST, D, B, MP and YT *(Serfass et al., 2006).* However, a limitation

of the present work was that the reference sample group was relatively young. In studies 1 and 2, the median age ranged from 2.3 to 4.2 years depending on the breed, whereas in study 3, median age of CKCS was 3 years. One of the main reasons for such a limitation is that prevalence of DMVD increases dramatically with age and may reach 100% in geriatric CKCS populations *(Beardow and Buchanan, 1993; Häggström et al., 1992; Chetboul et al., 2004a)*. It was therefore challenging to find old unaffected small size dogs. Nevertheless, the range of age was relatively representative of the small size dog population seen in routine veterinary practice. The sex ratio (male:female) was well-balanced in study 3 (ratio=0.86), whereas it was 4:6 in studies 1 and 2 which could be explained by the fact that all the dogs came from breeders.

Definition of healthy status

As for all the studies on RI determination, one important prerequisite for selection of the reference sample group was to define health criteria. In human beings, *The World Health Organization (Anonymous, 1946)* defines health as a *"complete physical, mental and social well-being."* Because this definition is not applicable to animals, the ASVCP Quality Assurance and Laboratory Standards Committee *(Friedrichs et al., 2012; ASVCP, 2012)* provided recommendations to define a reference population and verify the healthy status of reference individuals according to a study by *Walton (2001),* in which a non-exhaustive list of selection criteria was drawn up in order to help investigators for individuals' selection. These criteria were as follows: biological (age, gender, breed, stage of reproductive cycle, production type); clinical (history and physical examination including BCS); geographical (location) and seasonal (effects of temperature and day length). Finally, it had been noticed in the *ASVCP* recommendations that additional testing may be required to establish the health criteria, depending on the intended use

of the proposed RI *(Friedrichs et al., 2012; ASVCP, 2012).* In the studies performed here, we applied the former recommendations without requiring any additional testing because we believed that the healthy status of individuals could be determined according to a combination of complete history and physical examination. Since all the recruited dogs were predisposed to DMVD (which is characterized by a heart murmur), an important step to define the health criteria was to rule out this heart disease. Therefore, physical examination included a careful cardiac auscultation performed by a trained observer with a 3-year experience in cardiology. Finally, a quite large healthy population of adult small size dogs was directly selected within the general canine population with strict criteria of inclusion and exclusion in order to minimize the need for partitioning RI into criteria such as size, pregnancy/lactation, BCS, geographic location and circadian rhythm. Additionally, the reference sample group appeared to be relatively representative of the small size dog population living in the Paris area.

Selection of variables

The echocardiographic and blood variables for which the RI were determined in the present study were selected because of their clinical usefulness in the direct and indirect assessment of DMVD. Firstly, the TTE is the gold-standard method for screening and evaluation of DMVD in dogs *(Chetboul and Tissier, 2012).* In CKCS, the *French Spaniel Club* recommends that each CKCS ≥18 months old should undergo a mandatory echocardiographic screening to pass the confirmation examination. Using a specific TTE view (i.e., right parasternal 5-chamber view at the end of the T-wave on the ECG), presence or absence of DMVD is noted and mitral valve prolapse is graded from 0 (absence) to 4 (severe prolapse with *chordae tendineae* rupture). Moreover, in keeping with the ACVIM consensus statement on DMVD *(Atkins et al., 2009)*, each

CKCS, even those without a heart murmur, should regularly undergo a complete echocardiographic examination. The M-mode and pulsed-wave Doppler variables assessed in study 3 have been shown to be the most common variables used for the first-line evaluation of DMVD consequences, in order to detect left heart enlargement and myocardial dysfunction. Among the 15 tested echocardiographic variables, the LA/Ao ratio, the end-diastolic LVED and the mitral E wave have been shown to be associated with the clinical outcome of DMVD dogs (*Serres et al., 2007 & 2008; Borgarelli et al., 2008; Moonarmart et al., 2010; Reynolds et al., 2012; Hezzell et al., 2012a).*

Secondly, the use of the plasma cardiac biomarker NT-proBNP in combination with TTE has been advocated in many studies to assess DMVD severity, as well as response to medical therapy. This biomarker can also help clinicians to make a prognosis in both asymptomatic and symptomatic dogs with DMVD (*Boswood et al., 2008; Serres et al., 2008; Chetboul et al., 2009; Reynolds et al., 2012; Wolf et al., 2012 & 2013).* Finally, some plasma analytes may decrease (PCV, sodium, potassium and albumin) or increase (creatinine) during the course of DMVD, secondary to the development of CHF, but also secondary to administration of cardiac medications such as furosemide *(Farabaugh et al., 2004; Boswood et al., 2006; Nicolle et al., 2007; Boswood et al., 2009).* All these variables were therefore considered as the most relevant clinical variables to assess DMVD in small size breeds.

Study procedures

In the present work, all study procedures were standardized and had been previously described in sufficient details to allow reproduction by a trained staff. Additionally, all methods had been previously validated. Regarding assays for routine plasma biochemistry and plasma NT-proBNP,

a precise description of the analytical method was provided, including results of the intra- and inter-assay CV. Moreover, results for quality controls were also available for plasma biochemistry. However, one of the limitations of this work was that quality controls were performed using human samples although current ASVCP quality assurance guidelines recommend the use of specific species samples *(Flatland et al., 2010).* Furthermore, the stability of routine biochemistry analytes had been investigated in previous studies and known to be good in samples at room temperature and after freezing *(Thoresen et al., 1992 & 1995)*. To the contrary, little information was available regarding plasma NT-proBNP stability, representing another limitation of this work. Previous reports demonstrated that NT-proBNP concentration could be altered by sample handling and freezing. One study identified that NT-proBNP concentration was significantly greater in serum than in plasma of 53 healthy dogs *(Kellihan et al., 2009).* Additionally, NT-proBNP in EDTA plasma or serum taken from healthy dogs and dogs with heart disease showed a 50 to 51% decrease after 23-25 hours storage at room temperature *(Farace et al., 2008; Collins et al., 2010).* In the present work, a specific tube containing protease inhibitors helped limiting as much as possible degradation of NT-proBNP. One report showed that serum NT-proBNP increased significantly after freezing at -20°C to incorrectly group between 80 and 100% of normal dogs as having evidence of heart disease when using cut-off values suggested by some previous studies *(Collins et al., 2010).* An explanation raised by these authors was that freezing may have altered the morphology of the large proBNP molecule making more peptides available for measurement by the assay, or altered the protein structure so as to increase the affinity of the detection antibody for natriuretic antigen *(Collins et al., 2010).* However, stability of NT-proBNP at -80°C, i.e., the storage temperature in the present work remains unknown and should have been documented as recommended by the IFCC-CLSI.

Nevertheless, standardization of blood sampling and handling of blood specimen in this work allowed controlling as much as possible sources of pre-analytical and analytical variations. One of the major issues of this work was that limit of quantification of the NT-proBNP assay was 2842 pmol/L. The consequence was that 9 out of the 154 dogs (5.8%) included in study 2 had an observed value of plasma NT-proBNP greater than 2842 pmol/L. Their NT-proBNP value was therefore set as equal to 2842 pmol/L for statistical analysis. In 2013, a new generation of assay has been developed, allowing a limit of quantification of 10 000 pmol/L.

Procedures for cardiovascular examination (echocardiography and systemic arterial blood pressure measurement) were performed by trained observers (with at least 3 years of experience in cardiology), and had previously been described and validated *(Chetboul et al., 2004b; Chetboul et al., 2010)*. These conditions helped to minimize the influence of the observer's experience level and its own within-day and between-day variability. As an example, a study showed that for a trained observer, most of within-day and between-day intra-observer CV regarding measurements of 8 echocardiographic variables were acceptable, and ranged respectively, from 3.1% to 10.2% and from 4.1% to 25.8% *(Chetboul et al., 2004a)*.

Reference interval determination

The reference sample group comprised a sufficient number of observed values to allow RI determination by using the nonparametric method (i.e., 154 dogs in studies 1 and 2, and 134 CKCS in study 3). All RI were calculated thanks to a specific software *(Geffré et al., 2011)*, although the rank number method could have been applied, as illustrated in the manuscript for plasma sodium concentration (see page 57). One exception was for E and A mitral wave velocities and mitral E/A ratio (study 3), where only 101 observed values were available. In

keeping with the IFCC-CLSI that recommends at least 120 observations to apply the nonparametric method, and since these variables were not normally distributed, percentiles should have been estimated by using the nonparametric approach and 90% CI by resorting to the Bootstrap method, which represents a limitation of this work. An advantage of the nonparametric method was that non-Gaussian data did not need to be transformed to fit Gaussian distribution. Finally, as stated above, the limit of quantification of the NT-proBNP assay was relatively low (i.e., 2845 pmol/L) and the upper limit of the calculated RI corresponded to this limit. Therefore, the upper limit of the RI for plasma NT-proBNP in this small size dog population was not interpretable, which represent one of the major limitations of this work.

Identification and treatment of outlying observations

Identification of outliers was performed by using one of the statistical methods recommended by the IFCC-CLSI (i.e., the Tukey method). No outliers were identified in study 1, whereas in study 2, 11 outliers were detected (i.e., dogs with plasma NT-proBNP ≥2617 pmol/L). As the upper limit of the RI for plasma NT-proBNP corresponded to the limit of quantification of the assay (i.e., 2842 pmol/L), it was difficult to conclude if these 11 dogs were true outliers or not. Nevertheless, all these dogs underwent a complete cardiovascular examination, as well as assessment of plasma blood urea nitrogen and creatinine concentrations (since azotemia may increase plasma NT-proBNP concentrations, *Raffan et al., 2009*), 3 months after the first blood sampling. Additionally, study 3 revealed outlier dogs (n=3) for the maximal systolic pulmonary flow velocity variable. As stated in the material and methods section, we pre-determined criteria to decide whether to include or exclude outliers. These criteria were the following: any abnormalities about history, physical examination, TTE or systemic arterial blood pressure in

study 2, and any abnormality regarding physical examination and TTE in study 3. Since all outliers' examinations were considered normal, none of them were excluded for the RI determination. This was in accordance with the IFCC-CLSI, which recommends that elimination of outliers should not only be based on statistical concepts but also on a rational basis (i.e., absence of clinical evidence of illness).

Effect of covariates and need for partitioning

The effect of covariates such as breed, BW, age or gender was identified in all 3 studies by using a linear mixed-effect model. These covariates were chosen in the present work as they represent the most common covariates studied in veterinary medicine. In study 1, a breed, BW, age and gender effect was evidenced for 7/15, 8/15, 3/15 and 4/15 blood variables. In study 2, only a gender effect was identified for plasma NT-proBNP concentration, with females having higher median plasma NT-proBNP values than males. In study 3, a statistically significant effect of BW was observed for all variables except FS(%), LA/Ao ratio, mitral E/A ratio and heart rate. A significant effect of gender and age was also noticed for 5/15 and 4/15 of the tested echocardiographic variables, respectively. Partitioning criteria were presented in 1990 by *Harris and Boyd* who calculated the statistical significance of the difference between subclass means by the standard normal deviate z-test. The authors offered some specific recommendations for threshold or critical z-values to aid in deciding whether or not to calculate separate reference intervals *(Harris and Boyd, 1990).* This approach however assumes that the data follow a Gaussian distribution and take into account an unequal prevalence of subclasses *(Geffré et al., 2011).* An alternative method based on direct estimation of the proportion of two subclasses outside the reference limit at each end of the combined distribution has been proposed: *"If at*

least one of the four proportions of the subgroup distributions cut off by the reference limits of the combined distribution exceeds 4.1% or lies below 0.9%, subgroup-specific reference intervals should be calculated instead of using the common reference limits of the common group" (Lahti et al., 2004). More recently, it has been advocated that the decision to partition into sub-classes should not only be made on the basis of statistical procedures but also on a clinical point of view, that is, if physiologic differences are expected to result in important clinical differences in RI *(Kjelgaard-Hansen, 2010).* In the present work, we therefore discussed the clinical relevance of the effect of covariates for each variable. In study 1, plasma urea differed significantly according to the breed. Nevertheless, when the mean values were considered, only MP and ST dogs showed a 32% higher value than the other breeds. The higher values observed in these two breeds did not exceed the upper value of the 90% CI for the upper limit of the RI, suggesting therefore that the RI established from the overall population could be suitable for all 7 selected breeds. Moreover, in the same study, a significant BW effect was observed for half of the tested variables. When BW, for example, was decreased by 3 kg (which is a quite large difference within a given breed), plasma urea was decreased by 1.8 mmol/L in MP whereas it was increased by 2.4 mmol/L in YT. It was therefore conclude that these findings will have but little impact on the clinical interpretation of the results. In study 3, the clinical relevance of the effect of covariates was discussed with regard to observers' variability for TTE examination *(Chetboul et al., 2004b).* As an example, if BW was increased by 4 kg, the interventricular septum and the left ventricular free wall thicknesses was increased by 0.41 and 0.79 mm in diastole, and by 0.97 and 1.56 mm in systole, respectively. These differences were higher than the between-day SD assessed for a trained observer (i.e., 0.34 mm, 0.64 mm, 0.48 mm, and 1.34 mm, respectively). Similarly, for a 4-kg BW increase, the LVED and LVES were increased by 3.95 mm and 1.97 mm, respectively

(with corresponding between-day SD of 2.51 mm and 1.39 mm, respectively). Therefore, only the BW effect on left ventricular M-mode measurement was considered clinically relevant and the use of regression-based RI according to BW categories was performed *(Virtanen et al., 1998).* Conversely, the statistically significant effect of gender on LVED and LVES and the end-diastolic interventricular septum was considered as clinically meaningless, as demonstrated by the low slope coefficients (respectively, 0.641, 0.698, 0.178) in comparison to the corresponding between-day SD (respectively, 2.51, 1.39, 0.34). However, in study 2, although significantly higher values of plasma NT-proBNP were identified in females compared to males, RI according to gender were not determined. Hence, as stated above, the upper limit of the RI could not be interpreted as it corresponded to the limit of quantification of the NT-proBNP assay (i.e., 2842 pmol/L). Therefore, it was hypothesized that the difference in plasma NT-proBNP between males and females could partially be due to this analytical issue and partitioning was not undertaken.

The issue of individual variability

In study 2, plasma NT-proBNP concentrations in the global population displayed a wide range of values. Since we limited the sources of variability by standardizing the procedures for sample handling, storage and shipment of plasma samples, we therefore concluded that variability for plasma NT-proBNP was inter-individual rather than pre-analytical in this small size dog population. In the general population, the inter-individual CV was 96% and the within-breed inter-individual CV ranged from 62% (CKCS) to 100% (MP). When considering several other studies on plasma/serum NT-proBNP including groups of healthy dogs *(Oyama et al., 2008; Atkinson et al., 2009; Kellihan et al., 2009 & 2011; Raffan et al., 2009; Schmidt et al., 2009; Chetboul et al., 2009; Collins et al., 2010; Wess et al., 2011; Cunningham et al., 2012; Hezzell et*

al., 2012b), only two reports included healthy comparable breeds as in our, but had too small sample size (lower than 31 *versus* 154 in the present study) to allow any conclusion on inter-individual variability *(Chetboul et al., 2009; Hezzell et al., 2012b).* In one report including a higher number of dogs (n = 196) from the same large breed (Doberman Pinscher), inter-individual CV according to age category reached 53% in dogs ≤ 3 years *(Wess et al., 2011).* Study 2 was therefore the first one to point out the inter-individual variability of plasma NT-proBNP in small size dogs. However, according to the study by *Collins et al., 2010,* it was hypothesized that effect of sample handling and storage could have altered plasma NT-proBNP concentrations and explained this variability.

In study 1, a large inter-individual variability was also observed in the reference sample group for several variables, especially liver enzymes, but not for well-controlled variables (sodium for example). Enzymatic measurements are nevertheless known for their relatively high inter- and intra-individual variability *(Ruaux et al., 2012)*, but our findings for ALT here clearly need further investigation at least in B and MP as, to the best of the author's knowledge, no previous publication has reported such a high upper limit.

Clinical relevance of reference interval determination in small size dogs

The clinical utility of RI establishment in small size dogs was evaluated. In study 1, according to the analyte, 3.8% to 76.6% of the observed values were inferior to the lower limit of the laboratory's RI used routinely for 9 out of 12 tested variables, and 4.5% to 9.7% of the observed values were higher than the upper limit of the laboratory's RI for 7 out of 12 variables, thus indicating that the laboratory's RI was not appropriate for all variables except plasma glucose and chloride. These observations were already pointed out by other authors in adult dogs within a

specific breed such as Bernese Mountain dogs *(Nielsen et al., 2006)*, Greyhounds and Lurchers *(Campora et al., 2011a & 2011b)* as well as Dogues de Bordeaux *(Lavoué et al., 2013)* or category of breeds *(Craig et al., 2006).*

Regarding plasma NT-proBNP evaluation, study 2 was the first that tried to determine RI within a population of healthy dogs. High inter-individual variability was found within the studied population and led us to conclude that a single plasma value in a healthy adult small size dog should be interpreted carefully. Moreover, as for any laboratory test, many sources of variation may occur in the plasma NT-proBNP dosage, potentially leading to false-positive or false-negative results. Therefore, the TTE remains the method of choice in the assessment of heart disease and plasma NT-proBNP dosage should be performed in combination with echocardiography. Finally, due to an analytical issue, the upper limit of the RI corresponded to the limit of quantification of the assay and was therefore not interpretable. As NT-proBNP concentration is supposed to increase in dogs with DMVD, the real interest of the assay with such a low limit of quantification was pointed out.

Finally, in study 3, we compared all the M-mode echocardiographic RI with the predictive RI calculated using the Cornell's formula. The Cornell's formula *(Cornell et al., 2004)* is an allometric scaling allowing prediction of percentiles according to BW. It was developed from a compilation of various small and large breeds adult dogs originating from 12 previous studies. Although our RI were systematically comprised in the predictive percentiles calculated with the Cornell's formula, all were smaller than this latter, as illustrated by the end-diastolic left ventricular free wall (i.e., 5.0-8.3 mm in the present study *versus* 4.9-13.2 mm with Cornell's formula). This observation suggests that, according to *Morisson et al., 1992*, not only the BW,

but also the breed must be taken into account when determining RI for echocardiographic variables.

Conclusion

As a conclusion, the present results demonstrated that determination of specific RI in small size dogs is relevant regarding several blood and imaging variables because RI established from other canine breeds are not always suitable for small breeds. However, the need to partition RI for a specific small size breed was not identified for blood variables. These finding are of great clinical interest, not only for the longitudinal assessment of dogs with DMVD, but more widely, in other veterinary medicine specialties. Hence, veterinarians will now dispose of specific RI for routine biochemistry in the most common 7 small size breeds seen in practice. Moreover, effect of covariate such as BW, age and gender were observed in the present work but seemed to be negligible for biochemistry variables and PCV, and remain unclear for the plasma NT-proBNP. To the contrary, BW was strongly correlated with echocardiographic M-mode variables in CKCS, requiring regression-based RI according to range of BW. Finally, the plasma NT-proBNP is the most common cardiac biomarker used in veterinary medicine in the assessment of canine DMVD but also in the differential diagnosis of respiratory symptoms such as cough or dyspnea (i.e., cardiac or non-cardiac origin). However, due to high inter-individual variability observed in the present study, this useful biomarker should be interpreted with caution (especially high values) and in combination with echocardiography, which remains the gold standard of cardiovascular evaluation.

Perspectives

This work opens up new perspectives. Firstly, determination of RI for other cardiologic variables in small size dogs including troponin I, VHS and other echocardiographic variables (e.g., 3-dimensional, speckle tracking etc.) could be interesting. Moreover, RI for echocardiographic variables should be investigated in small size dogs other than CKCS, in order to confirm or infirm the need for determination of specific small breed-RI. As demonstrated here, the breed category should be taken into account when determining a RI for several blood and echocardiographic variables. Since morphological differences between canine breeds can be large, it is assumed that a RI should be ideally developed within a specific breed or category of breeds. This work will be huge as there are more than 300 breeds in the canine population but is however critical because it would probably avoid some mis-classifications of dogs with or without specific disease conditions. Moreover, the effects of BW, age and gender on the tested variables should be assessed when RI is being determined in order to know if further partitioning of is necessary. Additionally, evaluation of other effects such as training, pregnancy, BCS and neutered status could be highly relevant as only few studies reported their influence on blood and cardiovascular variables. However, according to *Kjelgaard-Hansen* and as demonstrated in the present work, partitioning should be undertaken only if a clinical relevance has been identified. Further studies are needed to better understand the concept of RI in the canine species and more widely in veterinary medicine.

APPENDIX

ARTICLE 1: "Basal plasma concentrations of routine variables and packed cell volume in clinically healthy adult small size dogs: effect of breed, body weight, age, and gender, and establishment of reference intervals"

This article was written by Misbach C, Chetboul V, Concordet D, Gruet P, Speranza C, Hoffmann AC, Rocha A, Balouka D, Petit AM, Trehiou-Sechi E, Pouchelon JL and Lefebvre HP and published in Veterinary Clinical Pathology in September 2014 (volume 43, pages 371 to 380).

ARTICLE 2: "Basal plasma concentrations of N-terminal pro-B-type natriuretic peptide in clinically healthy adult small size dogs: effect of breed, body weight, age, and gender, and establishment of reference intervals"

This article was written by Misbach C, Chetboul V, Concordet D, Gruet P, Speranza C, Hoffmann AC, Rocha A, Balouka D, Petit AM, Trehiou-Sechi E, Pouchelon JL and Lefebvre HP and published in Research in Veterinary Science in December 2013 (volume 95, pages 879 to 885).

ARTICLE 3: "Conventional and Doppler echocardiography in clinically healthy adult Cavalier King Charles Spaniels: effect of body weight, age, and gender, and establishment of reference intervals"

This article was written by Misbach C, Lefebvre HP, Concordet D, Gouni V, Trehiou-Sechi E, Petit AM, Damoiseaux C, Leverrier A, Pouchelon JL and Chetboul V and published in the Journal of Veterinary Cardiology in June 2014 (volume 16, pages 91 to 100).

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Veterinary Clinical Pathology ISSN 0275-6382

ORIGINAL RESEARCH

Basal plasma concentrations of routine variables and packed cell volume in clinically healthy adult small-sized dogs: effect of breed, body weight, age, and gender, and establishment of reference intervals

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Key Words
Albumin, canine, creatinine, partitioning, sodium, total proteins

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DOI:10.1111/vcp.12162

Background: Plasma variables may be affected by breed or body weight (BW). Small-sized dogs are very common, but no specific reference intervals (RI) are used.
Objectives: The primary objective of this prospective study was to assess the potential effect of breed, BW, age, and sex on routine plasma analytes and packed cell volume (PCV) in small-sized dogs. A secondary objective was to establish RI in this small-sized population.
Methods: Blood was sampled under standardized conditions from healthy dogs. PCV and 15 routine plasma variables were measured at the same laboratory. Effects of breed, BW, age, and sex were tested using a general linear model. The procedure recommended by the Clinical and Laboratory Standards Institute was used to establish RI.
Results: In this study, 154 healthy dogs from 7 breeds were prospectively included. Although a significant effect of breed, BW, sex, or age was evidenced for most variables (except plasma sodium, phosphates, and triglycerides), it was considered as clinically irrelevant. More strikingly, the percentage of values in the reference sample group under the lower limit of the laboratory's RI ranged from 3.8% to 76.6% for 9 variables, and those higher than the upper limit of the laboratory's RI ranged from 4.5% to 9.7% for 7 variables. For example, the RI for creatinine in small-sized dogs was 45–90 μmol/L (vs 54–144 μmol/L for the general dog population).
Conclusion: Specific RI should be considered for PCV and selected plasma variables in small-sized dogs.

Introduction

Breed effects on biochemical variables have been increasingly reported for dogs over the last 10 years[1–7], as illustrated for Sight Hound dogs.[8] The dog provides a unique model because there are > 300 breeds with large phenotypic variations. The decision to partition data according to breed might be valid not only for Sight Hounds but also for other breeds, as previously suggested.[9–12] Although significant differences were observed in Alaskan Malamute, Siberian Husky, Golden Retriever, and English Setter populations for various biochemical analytes, they were considered irrelevant by the authors.[13]

According to the American Kennel Club 2011 Dog Registration Statistics[14], small-sized dogs, ie, dogs with

a body weight (BW) < 12 kg, are popular. Yorkshire Terrier (YT), Poodle, Dachshund (D), and Shih-Tzu (ST), for example, rank 5, 8, 9, and 11, respectively, of 173 different canine breeds. These breeds are more common in large cities. The most popular breed in 2011 in Detroit, New York City, and Tampa was, for example, the YT. Therefore, specific reference intervals (RI) for small-sized dogs could be more appropriate in urban areas.

The phenotypic differences between small-breeds might also affect plasma and serum variables. Interestingly, limited data are available regarding the potential effect of BW on plasma variables, although this factor is probably confounded with the breed. The morphologic differences between canine breeds can be large with BW varying from < 1 kg (Chihuahua) to > 60 kg (Irish Wolfhound). This is particularly true for small-breed dogs as the BW range may vary more than 10-fold (from < 1 to 12 kg). For example, plasma creatinine can be significantly affected by BW in the canine species.[15,16] Effects of age and sex on most plasma variables are still poorly documented and could also affect clinical interpretation.

The primary objective of this prospective study was to assess the potential effect of breed on routine plasma analytes in dogs from 7 different small-breeds, and to assess the effects of BW, age, and sex on the plasma variables that were measured. A secondary objective was to use this small-sized dog population as a reference sample group to establish RI.

Material and Methods

Animals

The reference sample group consisted of healthy and sexually intact adult (age > 10 months and < 8 years) breeder-owned dogs of 7 different breeds, including Bichon Frisé (BF), Cavalier King Charles (CKC) and King Charles (KC) Spaniel, Miniature Poodle (MP), ST, YT, and D, prospectively recruited in the area of Paris, France. Breeder's consent was obtained for each animal before its enrollment in the study, and all procedures were approved by a local ethics committee and in compliance with the Procedures and Principles of Good Clinical Practice.[17] An animal information form (documenting breed, sex, date of birth, history, diet, reproductive status, vaccination program, and parasite control), and a copy of the pedigree were also obtained for each dog before inclusion in the study. Moreover, a body condition score (BCS) was determined for each dog based on a 9-point scale.[18] Specific attention was paid to avoid including dogs from the same family (eg, puppies from the same litter and parents), and a maximum of 10 dogs were recruited from a given breeder to avoid any bias due to breeder-dependent environmental effects (housing, diet, exercise, etc). Dogs of different breeds could be owned by the same breeder.

Animals were assessed as healthy on the basis of a complete physical examination and history. Past history of medical problems (eg, infectious disease, lameness, etc) was not an exclusion criterion if the animal had totally recovered and if the treatment had been stopped at least 3 months before the time of blood sampling.

Dogs had to be fasted for at least 10 h before blood sampling and the diet should not have been changed over the last 15 days. Females had to be nonlactating, not pregnant, and in anestrus.

Nonfasted dogs, dogs with abnormal clinical findings, and dogs on medication at the time of blood sampling were excluded from the study. Administration of antiparasitic drugs or vaccines during the 15 days preceding blood sampling was also an exclusion criterion.

Blood sample collection and specimen handling

All dogs were sampled within an 8-week period between July and August 2011, and during the same period of day between 10.00 AM and 2.00 PM, to avoid any potential circadian periodicity. Fasted dogs were acclimated to the investigator and the room for about 10 min just before sampling. Blood was drawn from the jugular vein of the unsedated animal in the same position (sitting) with a 20-Gauge needle (Neolus NN-2038S, Terumo Europe, Leuven, Belgium) and a 5-mL syringe (BD Discardit II H-905S, Becton Dickinson, Fraga, Espana). Three mL of blood were collected and divided into 2 plastic tubes, one containing K3-EDTA (Venosafe VF-052STK, Terumo France, Guyancourt, France), and the other Lithium Heparin (Venosafe VF-054SHL, Terumo France). Blood was centrifuged for 15 min at 1600*g* (EBA 20, Hettich, Tuttlingen, Germany) at room temperature within 30 min of blood sampling. The plasma was then pipetted into 2 separate Eppendorf tubes, and stored at 4°C before transfer to a −80°C freezer, less than 6 h after blood sampling. Plasma assays were performed once a week during the study period by the Laboratoire Vebiotel, Arcueil, France (LVA).

The packed cell volume (PCV) was determined on heparinized blood in a capillary tube immediately after blood sampling, by centrifuging for 5 min at 12,000*g* (Haemofuge 3522, Heraeus, MN, USA).

Assays

Details about the analytical methods used for plasma biochemistry are provided in Table 1. Fifteen plasma analytes were measured: sodium, potassium, chloride, calcium, phosphate, urea, creatinine, total proteins, albumin, glucose, cholesterol, and triglycerides concentrations, and activities of aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). Electrolytes and substrates/enzymes were determined by direct potentiometry with specific electrodes, and absorption spectrophotometry and colorimetric methods, respectively (Konelab 60, Thermo Clinical – 95, Cergy Pontoise, France).

Statistical analysis

Identification of outliers and determination of RI were performed according to the current Clinical and Laboratory Standards Institute guidelines.[19] Native and Box–Cox transformed data were first tested for normality by use of the Anderson–Darling test. When the data distribution remained non-Gaussian after Box–Cox transformation, the values in both tails of the distribution were examined visually. Outliers were identified by applying the Tukey method. When a value was deemed questionable, the following criteria were used to decide whether to remove a dog from the study: any abnormality concerning medical history or clinical examination and an atypical value for more than one analyte from the same dog. RI were defined as central 95% intervals bounded by the 2.5th and 97.5th percentiles. Upper and lower limits of RI with their 90% confidence intervals (CI) were determined in the global population by a nonparametric approach.[20]

Effects of breed and other covariates on plasma variables were tested using a statistical software package (Systat version 8.0, SPSS Inc, Chicago, IL, USA). A value of $P < .05$ was considered significant. Age and BW in each breed were compared by ANOVA. The effect of breed, sex, age, and BW on plasma variables was tested by the following linear mixed-effects model:

$$Y = \mu + \text{Breed} + \text{Sex} + a\text{Age} + b\text{BW} + (\text{Breed} \times \text{Age}) + (\text{Breed} \times \text{BW}) + (\text{Breed} \times \text{Sex}) + \varepsilon$$

where Y is the value of the plasma variable, μ is a constant term, age and BW are continuous variables, and a and b are the slope coefficients for age and BW irrespective of the breed. The other terms denote interactions between breed and age, breed and BW, and breed and sex. ε is the residual term of the model.

Results

Population

Of the 183 dogs selected by breeders for examination during the kennel visits, 29 were excluded from blood sampling collection: 12 of these dogs were presented with a heart murmur, 5 females were in estrus, 5 dogs

Table 1. Analytical methods for plasma analytes measured in a population of 154 healthy small-sized dogs, and between-run coefficients of variation (CV) for human control solutions (Abtrol, Thermo Electron Co, Madison, WI, USA and Nortrol, Thermo Electron Co, Madison, WI, USA, respectively).

Analyte	Methodology	Control Concentration 1	Between-Run CV (%)	Control Concentration 2	Between-Run CV (%)
Urea (mmol/L)	Colorimetric (urease and glutamate dehydrogenase)	14.8	3.90	6.5	3.22
Creatinine (μmol/L)	Colorimetric (modified Jaffe: kinetic measurement)	337	4.49	156	4.37
Total proteins (g/L)	Colorimetric (Biuret method)	45.8	2.73	63.1	2.41
Albumin (g/L)	Colorimetric (bromocresol green)	28.6	2.16	41.3	1.97
Glucose (mmol/L)	Colorimetric (glucose oxidase/peroxidase)	15.9	1.92	5.0	2.05
ALT (U/L)	Absorption spectrophotometry (alanine/oxoglutarate) without pyridoxal phosphate (30°C)	157	3.13	40	5.07
AST (U/L)	Absorption spectrophotometry (L aspartate/glutarate) without pyridoxal phosphate (30°C)	158	2.46	36	2.30
ALP (U/L)	Absorption spectrophotometry (P nitro phenylphosphate) (30°C)	275	5.10	80	6.90
Sodium (mmol/L)	Potentiometric (ion-selective electrode)	152	1.38	137	1.10
Potassium (mmol/L)	Potentiometric (ion-selective electrode)	6.5	1.92	4.2	1.85
Chloride (mmol/L)	Potentiometric (ion-selective electrode)	117	1.82	100	2.02
Calcium (mmol/L)	Colorimetric (arsenazo III)	3.18	2.81	2.25	2.15
Phosphate (mmol/L)	Colorimetric (molybdate ammonium)	2.04	4.16	1.03	5.87
Cholesterol (mmol/L)	Colorimetric (cholesterol esterase)	6.79	1.83	4.28	2.36
Triglycerides (mmol/L)	Colorimetric (lipase/peroxidase)	2.09	3.20	0.75	3.30

had been vaccinated 2 days before examination, 3 dogs were nonfasted, 2 females had pseudolactation, one dog had hyperthermia with severe neck dermatitis, and one dog was obese (BCS of 7/9).

The final dog population included 154 dogs belonging to 28 different breeders (see characteristics in Table 2). According to a 9-point BCS scale, body score was 4/9 (ie, slim) for 2 dogs (1.2%), 6/9 (ie, slightly overweight) for 11 dogs (7.1%), and 5/9 (ie, optimal) for the 141 remaining dogs (91.5%).

Breed effect

Descriptive statistics of plasma analytes and PCV for each breed and for the reference sample group are given in Table 3. Significant differences between breeds were evident for BW ($P < .001$), as expected, but not for age ($P = .411$). The difference in BW was greatest between CKC and YT (8.3 ± 1.4 and 3.0 ± 0.7 kg, respectively). Some interactions between breed and other covariates (BW, sex, but not age) were statistically significant (Tables 4–6). A breed effect was evident for PCV ($P = .032$), plasma urea ($P = .035$), total protein ($P = .019$), albumin ($P = .032$), glucose ($P = .003$), and chloride ($P = .026$) concentration, and ALT activity ($P = .002$).

Effects of BW, sex, and age on plasma variables

A statistically significant effect of BW was observed for PCV ($P = .042$), plasma urea ($P = .046$), creatinine ($P = .021$), total protein ($P = .001$), albumin ($P = .007$), cholesterol ($P < .001$), and calcium ($P = .028$) concentration, and ALP ($P = .027$) activity. A statistically significant sex effect was observed for potassium ($P = 0.002$) and cholesterol ($P = .033$) concentration, and plasma ALT ($P = .027$) and AST ($P = .013$) activities. Total protein ($P < .001$), potassium ($P = .031$), and calcium ($P = .001$) concentrations were significantly affected by age (Tables 4–6).

Determination of reference intervals

Box–Cox transformation yielded a Gaussian distribution only for PCV, whereas 15 of the 16 distributions of plasma variables tested for normality could not be transformed to fit a Gaussian distribution (Table 7). Application of the Tukey method did not reveal any outliers and therefore no value was deleted for RI establishment.

The corresponding lower (LL) and upper (UL) limits of the RI for tested plasma variables with 90% CI are provided in Table 7. For each variable, the LL and UL of the laboratory LVA are also given. The 90% CI upper value for the LL of the RI established from the data obtained in the 154 dogs was lower than the LL of the general dog RI of the laboratory for total protein, albumin, sodium, potassium, and calcium concentrations. The 90% CI lower value of the UL of the RI in the small-sized dogs was higher than the UL of the laboratory's RI for plasma phosphate and cholesterol concentrations, and ALT and AST activities. The number and percentage of dogs with values lower (higher) than the LL (UL) of the laboratory are given in Table 7.

Discussion

This study demonstrates a breed, BW, sex, and age effect for 7/16, 8/16, 4/16, and 3/16 tested variables, respectively. None of these covariates significantly affected plasma sodium, phosphates, or triglyceride concentrations. This study also demonstrates that commonly used RI of plasma variables could potentially lead to misinterpretation of values obtained in small-sized dogs.

The major advantages of this study were that (1) it was prospective with well-standardized conditions (fasting, standardized blood sampling procedure, assays performed in the same laboratory), (2) the total number of animals included was high enough to allow

Table 2. Represented breeds, sex, age, and body weight (BW) in the study population of 154 healthy small-sized dogs.

Breeds	Number of Dogs (%)	Number of Breeders*	Numbers for Each Sex (%)		Median Age (Min–Max) (Years)	Median BW (Min–Max) (kg)
			Females	Males		
Total	154	28	96 (62.3)	58 (37.7)	3.2 (0.7–9.1)	6.5 (1.9–12.3)
Bichon Frisé	8 (5.2)	2	4 (4.2)	4 (6.9)	4.2 (1.5–6.3)	4.4 (2.5–7.5)
Cavalier King Charles Spaniel	36 (23.4)	8	21 (21.9)	15 (25.9)	2.3 (1.1–8.8)	8.5 (5.4–11.4)
King Charles Spaniel	17 (11.0)	4	14 (14.6)	3 (5.2)	3.7 (0.9–7.7)	7.5 (6.1–8.8)
Dachshund	27 (17.5)	4	15 (15.6)	12 (20.7)	3.0 (1.0–8.7)	5.0 (3.7–12.3)
Miniature Poodle	20 (13.0)	5	12 (12.5)	8 (13.8)	3.4 (1.0–7.2)	5.5 (2.9–9.2)
Shih-Tzu	28 (18.2)	5	17 (17.7)	11 (19.0)	3.5 (0.7–8.4)	6.5 (4.2–11.9)
Yorkshire Terrier	18 (11.7)	5	13 (13.5)	5 (8.6)	3.9 (1.5–9.1)	3.2 (1.9–4.2)

*Animals from different breeds could belong to the same breeder.

Table 3. Breed-specific reference intervals for PCV and select plasma analytes in a population of 154 healthy small-sized dogs.

		Breed						
Variable	Global	Bichon Frisé	Cavalier King Charles Spaniel	King Charles Spaniel	Dachshund	Miniature Poodle	Shih-Tzu	Yorkshire Terrier
Packed cell volume (%)	44 (34–57)	45 (40–51)	42 (34–54)	43 (35–52)	46 (41–56)	49 (37–57)	45 (37–53)	44 (39–55)
Urea (mmol/L)	5.0 (3.3–13.3)	5.0 (3.3–6.6)	5.0 (3.3–8.3)	5.0 (3.3–8.3)	5.0 (3.3–8.3)	6.6 (3.3–13.3)	6.6 (3.3–13.3)	5.0 (3.3–8.3)
Creatinine (µmol/L)	63 (45–99)	63 (54–81)	72 (54–90)	63 (54–81)	54 (45–72)	72 (54–81)	63 (45–99)	63 (54–81)
Glucose (mmol/L)	5.6 (3.4–7.3)	5.0 (3.4–6.2)	5.0 (4.5–6.2)	5.0 (4.5–5.6)	5.6 (3.9–7.3)	5.0 (3.9–5.6)	5.0 (4.5–6.2)	5.0 (3.4–6.2)
Proteins (g/L)	56 (43–70)	55 (51–58)	53 (43–60)	53 (50–61)	58 (49–70)	57 (49–63)	58 (45–62)	60 (52–68)
Albumin (g/L)	28 (22–34)	29 (24–31)	28 (23–32)	28 (25–31)	27 (22–34)	29 (26–33)	28 (24–32)	28 (24–34)
Aminotransferase (U/L)	39 (4–296)	62 (26–277)	35 (19–182)	42 (29–74)	35 (18–125)	48 (14–294)	47 (26–296)	28 (4–69)
Aspartate aminotransferase (U/L)	25 (2–129)	28 (2–65)	21 (13–56)	22 (3–34)	27 (10–36)	31 (22–129)	28 (15–59)	20 (3–59)
Alkaline phosphatase (U/L)	26 (1–113)	43 (20–91)	19 (7–113)	26 (11–47)	28 (14–52)	27 (7—103)	31 (1–99)	30 (13–53)
Cholesterol (mmol/L)	4.39 (1.29–8.26)	3.87 (2.58–4.64)	4.39 (1.29–6.71)	4.90 (4.13–7.48)	3.35 (1.55–5.42)	4.39 (3.35–6.19)	5.42 (2.84–8.26)	3.61 (2.32–5.68)
Triglycerides (mmol/L)	0.57 (0.23–5.24)	0.57 (0.34–1.37)	0.46 (0.23–1.60)	0.68 (0.46–1.37)	0.46 (0.23–4.22)	0.57 (0.34–2.51)	0.68 (0.34–5.24)	0.57 (0.23–0.34)
Sodium (mmol/L)	146 (132–154)	150 (141–151)	145 (132–151)	147 (138–152)	143 (137–154)	150 (142–152)	144 (141–154)	144 (141–153)
Potassium (mmol/L)	4 (3.3–5.2)	3.9 (3.6–4.2)	4 (3.5–4.5)	3.8 (3.3–4.3)	3.9 (3.5–4.6)	4 (3.3–4.7)	4.1 (3.6–5.2)	4 (3.5–4.5)
Chloride (mmol/L)	111 (102–119)	112 (109–115)	110 (102–119)	109 (107–113)	110 (106–115)	113 (109–115)	110 (106–114)	112 (109–115)
Calcium (mmol/L)	2.4 (2.0–3.3)	2.4 (2.3–2.5)	2.4 (2.1–2.7)	2.5 (2.3–2.7)	2.3 (2.0–3.3)	2.4 (2.3–2.6)	2.5 (2.1–2.6)	2.4 (2.2–2.7)
Phosphates (mmol/L)	1.2 (0.5–2.9)	1.2 (0.7–1.6)	1.4 (0.9–1.8)	1.3 (0.8–1.6)	1.3 (0.8–1.9)	1.1 (0.8–1.6)	1.3 (0.8–2.9)	1.0 (0.6–1.5)

Data are median (Min–Max).

calculation of the RI according to the current CLSI guidelines, and (3) the age (0.7–9.1 years) and BW (1.9–12.3 kg) ranges were representative of the small-sized dog population seen in routine veterinary practice. The potential limitations were the differing numbers of represented breeds. The actual proportion of each breed in the total canine population, however, is not well balanced, but rather varies according to the country and also to urban or rural areas. The reference sample group of this study appears representative of the small-sized dog population living in the Paris area. However, the number of dogs per breed (8–36) is too low to allow any extrapolation of the present results to the global population of each breed.

Although a male: female sex ratio of 1:1 would have been ideal, the sex ratio in the present study was 4:6, which could be explained by the fact that all the dogs belonged to breeders.

Another limitation of this study were the quality controls that were based on human material, which is not compliant with the current ASVCP quality assurance guidelines recommending the use of species-specific quality control materials.[21] This issue should be addressed in future studies establishing breed-specific RI.

The objective of this study was to assess the potential need for partitioning the reference sample group according to covariables (breed, BW, age, and sex). Nevertheless, the decision to partition into subclasses should not only be made on the basis of statistical concepts but also with physiologic differences expected to result in important clinical differences in RI in mind.[22] In the present study, a small-sized dog population was preselected among the general canine population with strict criteria of inclusion, as well as standardized and controlled preanalytical and analytical procedures to minimize the need for partitioning RI according to criteria, such as breed size (eg, small, large, and giant breed), pregnancy/lactation, reproductive status, geographic location, and circadian rhythm. Nevertheless, a large variability was observed in the reference sample group for several variables, especially liver enzymes, but not for physiologically well-regulated variables, such as sodium. This is also illustrated by the 90% CI of the limits of the RI. Currently, the high variability remains unexplained.

The clinical relevance of the statistically significant differences due to the different covariates should therefore be discussed for each variable. Plasma urea concentration differed significantly according to the breed. Nevertheless, when the mean values were considered, only MP and ST dogs showed a 32% higher value than the other breeds. The higher values

Table 4. Slope coefficients for the continuous variables age and body weight, and differential effects for the categorical variables breed and sex in the model used for statistical analysis of select plasma variables in a population of 154 healthy small-sized dogs, with corresponding *P*-values. The slope coefficients and differential effects are only given when the effect is statistically significant.

		Breed		Sex		Age		Body Weight	
Tested Variable	Mean of Entire Study Population	Differential Effect	*P*-Value	Differential Effect	*P*-Value	Slope Coefficient	*P*-Value	Slope Coefficient	*P*-Value
Urea (mmol/L)	7.3	BF: −1.6 CKC: +1.6 KC: −2.3 D: −0.9 MP: +4.5 ST: −2.6 YT: +1.2	.035	–	.58	–	.63	BF: +0.1328 CKC: −0.4316 KC: −0.1162 D: −0.1992 MP: −0.6142 ST: +0.4316 YT: +0.7968	.046
Creatinine (μmol/L)	60	–	0.14	–	.25	–	.07	+1.935	.021
Total protein (g/L)	50.080	BF: +1.660 CKC: −7.722 KC: −4.368 D: −2.667 MP: +6.108 ST: +7.390 YT: −0.351	.019	–	.196	+0.869	< .001	BF: −0.592 CKC: +1.013 KC: +1.030 D: +1.533 MP: −0.331 ST: −0.670 YT: −1.983	.001
Albumin (g/L)	25.933	BF: −3.092 CKC: −1.681 KC: −4.677 D: +2.485 MP: +5.617 ST: +0.005 YT: +1.343	.032	–	.899	–	.072	BF: +0.657 CKC: +0.623 KC: +0.433 D: −0.679 MP: −0.873 ST: −0.156 YT: +0.595	.007
Glucose (mmol/L)	4.9	BF: −0.6 CKC: +0.8 KC: +1.7 D: +0.9 MP: −0.3 ST: −0.3 YT: −2.2	.003	–	.22	–	.56	–	.15

BF indicates Bichon Frisé; CKC, Cavalier King Charles Spaniel; KC, King Charles Spaniel; D, Dachshund; MP, Miniature Poodle; ST, Shih-Tzu; YT, Yorkshire Terrier.

The general regression equation was calculated as follows (eg, plasma albumin in CKC): Plasma albumin (g/L) = 25.933 − 1.681 + 0.623 × Age (years).

observed in these 2 breeds did not exceed the upper value of the 90% CI for the UL of the RI, suggesting therefore that the RI established from the overall population could be suitable for all 7 selected breeds. For ALT activity, values > 80 U/L (the upper limit of the laboratory's RI) were observed in 15 dogs. This explains why the upper limit of the RI for ALT activity is quite high (261 U/L). The breed effect was statistically significant with BF dogs having the highest mean value. This could be explained by the fact that 2/8 BF dogs had high ALT activities (133 and 277 U/L). Similarly, 4/20 MP showed ALT activities between 115 and 294 U/L. The reason for such high activities remains unclear as these animals were clinically healthy and did not show any abnormality. These values were therefore kept for the RI determination. Nevertheless, as enzymatic activity measurements are known for their relatively high inter- and intra-individual variability, the ALT activities determined in this study for BF and MP require further investigation, as, to the best of the author's knowledge, no previous publication has reported such a high upper limit. Differences between breeds, although significant, appear to be clinically irrelevant for PCV, and plasma total protein, albumin, glucose, and chloride concentrations.

A significant BW effect was observed for half of the tested variables. Moreover, this effect differed according to the breed for plasma urea, total protein, and albumin concentration, and ALP activity. For example, when BW was decreased by 3 kg (which is a considerable difference within a given breed), plasma urea concentration was lowered by 1.8 mmol/L in MP,

Table 5. Slope coefficients for the continuous variables age and body weight, and differential effects for the categorical variables breed and sex of the model used for statistical analysis of PCV, plasma cholesterol concentration, and ALT and AST activities in a population of 154 healthy small-sized dogs, with corresponding *P*-values. The slope coefficients and differential effects are only given when the effect is statistically significant.

Tested Variable	Mean of Entire Study Population	Breed		Sex		Age		Body Weight	
		Differential Effect	*P*-Value	Differential Effect	*P*-Value	Slope Coefficient	*P*-Value	Slope Coefficient	*P*-Value
Packed cell volume (%)	39.8	BF: +8.4 CKC: +1.1 KC: −18.5 D: +5.1 MP: +14.0 ST: −1.6 YT: −8.5	.032	–	.426	–	.971	+0.894	.042
Aminotransferase (U/L)	84	BF: +133 CKC: +14 KC: −115 D: −59 MP: +89 ST: −44 YT: −18	.002	BF: F: −38, M: 38 CKC: F: +13, M: −13 KC: F: +17, M: −17 D: F: +6, M: −6 MP: F: −17, M: +17 ST: F: +4, M: −4 YT: F: +16, M: −16	.027	–	.198	–	.296
Aspartate aminotransferase (U/L)	36	–	.391	F: −3, M: +3	.013	–	.498	–	.166
Alkaline phosphatase (U/L)	33	–	.182	–	.761	–	.824	BF: −7.193 CKC: −5.330 KC: +0.310 D: −0.632 MP: +2.200 ST: +6.984 YT: +3.661	.027
Cholesterol (mmol/L)	2.6	–	.34	BF: F: +0.59, M: −0.59 CKC: F: +0.82, M: −0.82 KC: F: −0.05, M: +0.05 D: F: −0.45, M: +0.45 MP: F: −0.29, M: +0.29 ST: F: −0.18, M: +0.18 YT: F: −0.44, M: +0.44	.033	–	.76	+0.137	< .001

BF indicates Bichon Frisé; CKC, Cavalier King Charles Spaniel; KC, King Charles Spaniel; D, Dachshund; MP, Miniature Poodle; ST, Shih-Tzu; YT, Yorkshire Terrier.

The general regression equation for PCV was calculated as follows (eg, in Bichon Frisé): PCV (%) = 39.8 + 8.4 + 0.894 × Age (years).

whereas it was increased by 2.4 mmol/L in YT. Such findings will have little effect on the clinical interpretation of the results. The underlying mechanisms for this breed-dependent effect of BW cannot be explained, but could be related more to differences in metabolism rather than in renal excretion as this effect was not observed for plasma creatinine. The effect of BW on PCV, and plasma creatinine, calcium, and cholesterol concentration is negligible as shown by the low value of the BW slope coefficients. Therefore, a difference of BW from 2 to 12 kg (ie, a 6-fold variation) between 2 small-sized dogs was related to an increase of about 9% for PCV, and 19 µmol/L for plasma creatinine, 0.28 mmol/L for calcium, and 1.3 mmol/L for cholesterol concentration. Similar trends were seen for total plasma protein and albumin concentrations. For ALP activity, the slope coefficients were higher, indicating that a 3 kg difference in BW was related to a change in plasma enzyme activity of about 20 U/L in BF or ST. Based on the small magnitudes of the differences found, it can be assumed that changes in BW will not affect the clinical decision for a tested variable within the reference sample group.

A sex effect was evidenced for potassium and cholesterol concentrations, and ALT and AST activities. A significant interaction between breed and sex was observed for plasma cholesterol concentration and ALT activity, indicating that the sex effect was dependent on the breed. Conflicting results regarding the sex effect on plasma or serum variables have been published by different authors.[23–27] The sex effect could be breed-specific as seen here with much higher ALT

Table 6. Slope coefficients for the continuous variables age and body weight, and differential effects for the categorical variables breed and sex of the model used for statistical analysis of the plasma potassium, chloride, and calcium in a population of 154 healthy small-sized dogs, with corresponding *P*-values. The slope coefficients and differential effects are only given when the effect is statistically significant.

		Breed		Sex		Age		Body Weight	
Tested Variable	Mean of Entire Study Population	Differential Effect	*P*-Value	Differential Effect	*P*-Value	Slope Coefficient	*P*-Value	Slope Coefficient	*P*-Value
Potassium (mmol/L)	4.0	–	.641	F: −0.09 M: +0.09	.002	+0.029	.031	–	.246
Chloride (mmol/L)	110	BF: −1.9 CKC: −1.4 KC: −1.6 D: −2.0 MP: +2.4 ST: −3.1 YT: 7.5	.026	–	.146	–	.655	–	.459
Calcium (mmol/L)	2.41	–	.28	–	.53	−0.025	.001	+0.028	.028

BF indicates Bichon Frisé; CKC, Cavalier King Charles Spaniel; KC, King Charles Spaniel; D, Dachshund; MP, Miniature Poodle; ST, Shih-Tzu; YT, Yorkshire Terrier.

The general regression equation for calcium was as follows: Calcium (mmol/L) = 2.41 − 0.025 × Age (years) + 0.028 × body weight (kg).

Table 7. Reference intervals established for a study population of 154 healthy small-sized dogs and comparison with the reference intervals of the laboratory (Laboratoire Vébiotel, Arcueil, France) determined in a healthy general canine population.

Analyte	Distribution	LL (90% CI)	UL (90% CI)	LL–UL of the Laboratory	Number (%) of Values < LL of the Laboratory	Number (%) of Values > LL of the Laboratory
Packed cell volume (%)	BCG	35.9 (34–37)	56 (54–57)	37–55	6 (3.8%)	3 (1.9%)
Urea (mmol/L)	NG	3.3 (3.3–3.3)	11.6 (8.3–13.3)	3.3–8.3	0	7 (4.5%)
Creatinine (μmol/L)	NG	45 (45–54)	90 (81–99)	54–144	44 (28.6%)	0
Total proteins (g/L)	NG	47.6 (43–49)	67.1 (65–70)	60–80	118 (76.6%)	0
Albumin (g/L)	NG	23.9 (22–24)	33.0 (32–34)	30–40	118 (76.6%)	0
Glucose (mmol/L)	NG	3.9 (3.4–3.9)	6.2 (6.2–7.3)	3.9–6.2	2 (1.3%)	2 (1.3%)
Aminotransferase (U/L)	NG	19 (4–22)	261 (133–296)	< 80	–	15 (9.7%)
Aspartate aminotransferase (U/L)	NG	3 (2–10)	66 (54–129)	10–50	6 (3.9%)	9 (5.8%)
Alkaline phosphatase (U/L)	NG	7 (1–10)	92 (76–113)	<80	–	7 (4.5%)
Sodium (mmol/L)	NG	137 (132–139)	152 (152–154)	145–160	69 (44.8%)	0
Potassium (mmol/L)	NG	3.5 (3.3–3.5)	4.6 (4.4–5.2)	3.6–5.8	11 (7.1%)	0
Chloride (mmol/L)	NG	106 (102–107)	115 (115–119)	95–115	0	1 (0.6%)
Calcium (mmol/L)	NG	2.12 (2.00–2.20)	2.67 (2.62–3.29)	2.25–2.99	9 (5.8%)	1 (0.6%)
Phosphate (mmol/L)	NG	0.74 (0.55–0.81)	2.65 (1.84–2.91)	0.81–1.61	6 (3.9%)	13 (8.4%)
Cholesterol (mmol/L)	NG	1.81 (1.29–2.32)	7.48 (6.71–8.26)	0.52–6.45	0	8 (5.2%)
Triglycerides (mmol/L)	NG	0.23 (0.23–0.34)	2.51 (1.60–5.24)	0.23–1.71	0	6 (3.8%)

BCG indicates Gaussian after Box–Cox transformation; NG, non-Gaussian; LL, lower limit; UL, upper limit.

activities in male BF and MP dogs compared with female dogs, as reported previously in Beagles.[25] Our results obtained for the large overall small-sized population tested here showed nevertheless that there is no need to establish sex-specific RI for most tested variables.

A significant positive effect of age was observed on total protein and potassium concentration, and a negative effect on calcium concentration. No interaction between age and breed was observed here, contrary to results obtained in Beagles and Labrador Retrievers.[2] The slope coefficients were +0.869 for total protein, +0.029 for potassium, and −0.025 for calcium concentration. Therefore, a 5-year difference in age between 2 dogs is related to a change of +4.3 g/L in total protein, +0.15 mmol/L in potassium, and −0.13 mmol/L in calcium concentration in plasma. An increase in total serum proteins with age has previously been observed.[24] In Beagles and Labrador Retrievers, the plasma calcium concentration also decreases with age,

but the total plasma protein concentration remains stable.[2] These changes are again clinically not relevant enough to justify the establishment of RI based on age categories, except perhaps in growing or geriatric animals for which further studies would be needed to address this issue. In Beagles and Labrador Retrievers, the hematocrit, calcium, phosphorus, and protein concentrations, and ALP activity were indeed shown to have age-specific ranges within or during the first year of life.[2]

It can therefore be assumed that there is no need to establish breed-, sex-, age-, or BW-specific RI for most of the variables in the 7 small-breeds tested here, although further studies are needed to clearly identify breed and BW effects on the tested plasma variables. More interestingly, depending on the analyte, 3.8–76.6% of the observed values were lower than the LL of the laboratory's RI used routinely for 9/12 tested variables, and 4.5–9.7% of the observed values were higher than the UL of the laboratory's RI for 7/12 tested variables, thus indicating that the laboratory's RI (established for a general dog population) was not appropriate for all variables except for plasma glucose and chloride concentrations. Partitioning of RI is a critical issue in terms of statistical and clinical significance.[19] Partitioning criteria were presented earlier where the statistical significance of the difference between subclass means by the standard normal deviate (z) test was calculated.[28] The authors offered some specific recommendations for threshold or critical z-values to aid in deciding whether or not to calculate separate RI.[28] This approach, however, assumes that the data follow a Gaussian distribution, and takes into account an unequal prevalence of subclasses.[19] An alternative method based on direct estimation of the proportion of 2 subclasses outside the reference limit at each end of the combined distribution has been proposed as follows: "If at least one of the four proportions of the subgroup distributions cut off by the reference limits of the combined distribution exceeds 4.1% or lies below 0.9%, subgroup-specific reference intervals should be calculated instead of using the common reference limits of the common group."[29] This approach has been applied recently for the establishment of RI in feline breeds.[30] It is obvious from these partitioning criteria that specific RI in small-sized dogs rather than in the general canine population are needed for many variables, and especially for creatinine, total protein, albumin, and sodium concentration. For example, the use of a value of 144 μmol/L as the UL value for creatinine concentration will lead to false-negative results for the detection of renal dysfunction in small-sized dogs, as previously suggested.[16] Inversely, a normal albumin concentration could be falsely interpreted as hypoalbuminemia in small-sized dogs.

In conclusion, this study seems to indicate that the establishment of specific RI for routine variables in small-sized dogs rather than in the general canine population could be clinically relevant, especially in urban areas where this subgroup is highly represented. Inversely, the need for specific RI according to BW, sex, or age in small-sized dogs is questionable. However, further investigations are needed with a large number of dogs to clearly document a breed effect on plasma variables in the small-sized dog population.

Disclosure: The authors have indicated that they have no affiliations or financial involvement with any organization or entity with a financial interest in, or in financial competition with, the subject matter or materials discussed in this article.

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Research in Veterinary Science 95 (2013) 879–885

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Basal plasma concentrations of N-terminal pro-B-type natriuretic peptide in clinically healthy adult small size dogs: Effect of body weight, age, gender and breed, and reference intervals

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ARTICLE INFO

Article history:
Received 22 May 2013
Accepted 24 July 2013

Keywords:
Biomarker
Canine
Heart
Hormone
Natriuretic peptides

ABSTRACT

Plasma NT-proBNP has previously been evaluated in dogs with degenerative mitral valve disease (DMVD). However, reference intervals (RI) established according to the Clinical Laboratory and Standards Institute (CLSI) recommendations have never been provided. The objectives of this prospective study were to assess effects of breed, body weight, age, and sex on plasma NT-proBNP, and to establish RI according to CLSI for this biomarker in a large population of dogs predisposed to DMVD.

183 Healthy small-sized dogs from 7 breeds were included. Assays were performed by ELISA. Effects of covariates were tested using a general linear model. Although a sex effect was demonstrated ($P = 0.01$), no significant effect of breed, body weight or age was shown. The proposed RI was 157–2842 pmol/L. 7% of dogs had plasma NT-proBNP >2617 pmol/L, and were considered as outliers despite normal cardiovascular examination. In conclusion, plasma NT-proBNP may be high in a few healthy small-sized dogs.

1. Introduction

Degenerative mitral valve disease (DMVD) is the most common acquired heart disease in small size dogs, and is characterized by degenerative valvular lesions resulting in systolic mitral regurgitation with potential hemodynamic consequences, such as reduced forward cardiac output and increased intracardiac pressures (Kvart and Häggström, 2005; Borgarelli et al., 2008). As demonstrated in humans (Francis, 1998; Ferrari et al., 1998), such hemodynamic alterations may result in complex neurohormonal activation (especially adrenergic nervous and renin–angiotensin–aldosterone system activation with overexpression of natriuretic peptides), in order to maintain adequate cardiac output, blood pressure, and tissue perfusion. To date, natriuretic peptides, including the inactive aminoterminal portion of brain natriuretic peptide (N-terminal pro-B-type natriuretic peptide, NT-proBNP), are considered as one of the most reliable neurohormonal markers of heart diseases in dogs (Sisson, 2009; Boswood, 2009; Oyama, 2009a). Plasma NT-proBNP is correlated with canine DMVD severity, and can be used in combination with clinical status to predict outcome in both asymptomatic dogs and dogs with congestive heart failure (CHF) (Oyama et al., 2008; Serres et al., 2009; Chetboul et al., 2009; Reynolds et al., 2012). Moreover, this biomarker has also been used to discriminate between CHF and primary pulmonary disease in dogs with cough or dyspnea (Fine et al., 2008; Oyama et al., 2009b). Finally, plasma NT-proBNP has been shown to decrease with treatment of CHF (Atkinson et al., 2009; Schöber et al., 2011) and the reduction in plasma NT-proBNP after initiation of treatment is considered as a useful predictor of overall cardiac survival (Wolf et al., 2012). Plasma NT-proBNP has been previously evaluated in a large population of healthy dogs ($n = 550$), including small and large breed dogs from 9 different breeds (Häggström et al., 2012). Highly significant breed differences were found and the authors concluded that breed-specific reference ranges might therefore be necessary for optimal clinical use of natriuretic peptides as cardiac biomarkers.

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http://dx.doi.org/10.1016/j.rvsc.2013.07.025

However, to the best of the authors' knowledge, no study has specifically documented reference intervals (RI), nor the potential effects of physiological covariates (e.g., breed, body weight, age, and sex) on plasma NT-proBNP concentration in a large population of healthy adult small size dogs from different breeds predisposed to DMVD.

The aims of this prospective study were therefore (1) to evaluate plasma concentrations of NT-proBNP, (2) to identify potential effects of breed, body weight, age, and sex and (3) to establish tentative RI according to the statistical procedures recommended by the Clinical Laboratory and Standards Institute guidelines (CLSI, 2008), in a large population of healthy adult small size dogs from different breeds known to be predisposed to DMVD.

2. Material and methods

2.1. Animals

The study population consisted of healthy non-neutered adult (age >10 months and <8 years) dogs of 7 different breeds predisposed to DMVD (Bichon (B), Cavalier King Charles (CKC) and King Charles (KC) Spaniels, miniature Poodle (MP), Shih-Tzu (ST), Yorkshire Terrier (YT), and Dachshund (D)), prospectively recruited in the Paris area (France). Breeder's consent was obtained for each animal before its enrolment in the study and all procedures were approved by a local ethics committee and in compliance with the Procedures and Principles of Good Clinical Practice (Food and Drug Administration Good Clinical Practice, 2012). An animal information form (including breed, sex, date of birth, history, diet, and also reproductive, vaccination and deworming status) and a copy of the pedigree were obtained for each dog before inclusion. Dogs from different breeds could be owned by the same breeder. However, specific attention was paid to avoid including dogs from the same family (e.g., siblings, parents, and littermates), and a maximum of 10 dogs were recruited from a given breeder in order to avoid any bias due to breeder-dependent environmental effects (housing, diet, exercise, etc.).

Animals were assessed as healthy on the basis of a complete physical examination, history, and routine plasma biochemistry. Occurrence of clinical signs and past history of medical events (e.g. infectious disease, lameness, etc.) did not lead to exclusion of a dog from the study if the animal had totally recovered and if the treatment had been stopped at least 3 months before blood sampling. Occurrence of clinical signs between the time of blood collection and 2 months later was assessed by a phone call interview with the breeder.

Dogs had to be fasted for at least 10 h before sampling and the diet should not have been changed during the previous 15 days. Females had to be in anoestrus, and neither lactating nor pregnant.

Non-fasted dogs, overweight dogs, dogs with an abnormal clinical examination, and dogs on medication at the time of blood sampling were excluded from the study. Other exclusion criteria were antiparasitic drug administration or vaccination during the 15 days preceding blood sampling.

2.2. Blood sample collection

To avoid any potential circadian periodicity, blood was collected during the same period of the day (between 10.00 AM and 2.00 PM) and all the dogs were sampled within an 8-week period (July–August 2011). Fasted dogs were acclimated to the investigator and the room for 10 min before sampling. Blood was drawn with a 20 G needle and a 5 mL syringe from the jugular vein of awake animals, which were always in the same position (sitting position). Two mL of blood were collected and placed in a plastic tube containing K3-EDTA (Venosafe VF-052STK, Terumo France, Guyancourt, France). Blood was centrifuged (15 min, 1500*g*) (EBA 20, Hettich, Tuttlingen, Germany) at room temperature within 30 min of blood sampling. The plasma (at least 0.5 mL) was placed into transport tubes containing proteases (Cardiopet, Idexx, Alfort, France) provided by the commercial laboratory (Laboratoire Idexx, Alfort, France), transferred at 4 °C within 45 min of blood sampling and then stored at −80 °C (less than 6 h after blood sampling).

2.3. Plasma NT-proBNP assay

Plasma NT-proBNP concentration was measured using EDTA-potassium samples and a commercially available canine specific assay (Cardiopet, Idexx, Alfort, France). This sandwich ELISA assay has already been used and validated for diagnostic purposes in the dog (Boswood et al., 2008; Zieba et al., 2008). The inter- and intra-assay coefficients of variation (CV) were 5.3, 6.6, 9.2%, and 10.7, 2.1, 4.2% for low (600 pmol/L), medium (1200 pmol/L) and high (2400 pmol/L) concentrations, respectively. Samples were sent every two weeks to the commercial laboratory (Laboratoire Idexx, Alfort, France).

2.4. Statistical analysis

A value of $P < 0.05$ was considered significant. Identification of outliers and determination of RI were performed according to the current CLSI guidelines (2008). Native and Box-Cox transformed data were first tested for normality by use of the Anderson–Darling test. When the data distribution remained non-Gaussian after Box-Cox transformation, visual inspection of values in both tails of the distribution was used. Identification of outliers was performed using the Tukey method. When an outlier was identified, further examinations (see below) were specifically scheduled. The results of these examinations were used as criteria to decide whether an outlier dog should be removed or not from the study.

Effects of breed and other covariates on plasma NT-proBNP concentration were tested using a statistical software package (Systat version 8.0, SPSS Inc, Chicago, IL). Age and body weight in each breed were compared by ANOVA. The effects of breed, sex, age, and body weight on plasma NT-proBNP concentration were tested using the following linear mixed effects model:

$$Y = \mu + \text{Breed} + \text{Sex} + a\text{Age} + b\text{Body weight} + (\text{Breed} \times \text{Age}) + (\text{Breed} \times \text{Body weight}) + (\text{Breed} \times \text{Sex}) + \varepsilon,$$

where Y is the value of the plasma variable; μ is a constant term; a and b are the slope coefficients for age and body weight irrespective of the breed. The other terms denote interactions between breed and age, breed and body weight, and breed and sex. ε is the residual term of the model.

Reference intervals were defined as central 95% intervals bounded by the 2.5th and 97.5th percentiles. The upper and lower limits of the RI with their 90% confidence intervals (CI) were determined in the global population using a non-parametric approach (Geffré et al., 2011). Data were expressed as median, [interquartile ranges], except for results regarding systolic arterial blood pressure and plasma BUN and creatinine (mean ± SD and [ranges] as minimum and maximum) in outlier dogs.

2.5. Further examinations for NT-proBNP outliers

After the Tukey method was applied, some of the tested dogs appeared as outliers (see statistical analysis). A complete cardiovascular examination, including conventional echocardiography and Doppler examinations as well as systemic arterial blood pressure measurement and plasma NT-proBNP, blood urea nitrogen (BUN) and creatinine determinations were specifically scheduled for these outliers 3 months after the first assay.

Systolic arterial blood pressure was indirectly measured before each echocardiographic examination in conscious dogs, in accordance with the ACVIM consensus statement (Brown et al., 2007) by the same trained observer (CM) using the Doppler method (811-BL, Parks Medical Electronics Inc. Aloha, Ore, USA). Dogs were gently held by the owner in sternal recumbency. An inflatable cuff (Soft-cuf, Ref 2422, 2 cm large, Parks Medical, USA) of appropriate-size was placed on the tail, as previously described (Chetboul et al., 2010). A period of acclimatization was allowed for each patient before blood pressure was measured. Several measurements were performed over 5–10 min to obtain a stable set of 5 values, the mean of which was taken as the patient's systolic blood pressure.

Conventional echocardiographic and Doppler examinations were performed by the same trained observer (CM) in awake dogs gently restrained in standing position, using continuous ECG monitoring with an ultrasound unit (Vivid i BT 10 SW appl. R 10.3.0, GE Healthcare, 9900 Innovation Drive, Wauwatosa, WI 53226, USA) equipped with a 5S (2–5 MHz) phased-array transducer, as previously described (Chetboul et al., 2004, 2005). Echocardiographic variables included the left ventricular diameters, the left ventricular free wall and interventricular septum thicknesses at end-diastole and end-systole as well as the fractional shortening for M-mode, and the left atrium on aorta ratio for the two-dimensional mode. Conventional Doppler variables included the maximal systolic aortic and pulmonary velocities, maximal early (E) and late (A) diastolic mitral flow velocities, as well as systolic pulmonary arterial pressure and diastolic pulmonic artery-to-right ventricle pressure gradient, when tricuspid and pulmonary regurgitations were identified, respectively. Echocardiographic and Doppler variables were compared with the previously established reference ranges (Gonçalves et al., 2002; Chetboul et al., 2005).

3. Results

3.1. Population

Twenty-nine of the 183 dogs selected by breeders for examination during the kennel visit were excluded from blood sample collection. Twelve of these dogs presented with a heart murmur, 5 females were in estrus, 5 dogs had been vaccinated 2 days before examination, 3 dogs were non-fasted, 2 females were having a pseudolactation, one dog had hyperthermia with severe neck dermatitis, and one dog was overweight.

One hundred and fifty-four dogs belonging to 28 different breeders were therefore included in the study (see characteristics in Table 1). Significant differences between breeds were found for body weight ($P < 0.001$) as expected, but not for age ($P = 0.411$). The greatest difference in body weight was observed between CKC and YT (8.4 kg [7.8–8.9] and 3.2 kg [2.3–3.3], respectively).

3.2. Plasma NT-proBNP concentration

The commercial laboratory (Laboratoire Idexx, Alfort, France) did not quantify the NT-proBNP concentration when it exceeded 2842 pmol/L. In 9 out of the 154 tested dogs, plasma NT-proBNP exceeded 2842 pmol/L, so their NT-proBNP value was set at 2842 pmol/L for the statistical analysis.

The plasma NT-proBNP values obtained in the global tested population and in each of the 7 tested breeds are given in Table 2. Distribution of plasma NT-proBNP concentrations among the reference sample group is illustrated in Fig. 1. Although no age or breed effect was observed on plasma NT-proBNP, a significant sex effect ($P = 0.01$) was shown (males: 683 pmol/L [404–892]; females: 844 pmol/L [550–1327]).

3.3. Characteristics of NT-proBNP outliers

After the Tukey method was applied, 11 of the 154 dogs included in the study were considered as outliers (i.e., plasma NT-proBNP concentration $\geqslant$2617 pmol/L). The plasma NT-proBNP concentration was >2842 pmol/L for 9 dogs, and 2816 and 2829 pmol/L for the 2 remaining dogs. As described above, a complete cardiovascular examination, as well as plasma BUN and creatinine determination were specifically scheduled 3 months after the first blood sampling.

The outlier population (8 females and 3 males, age: 3.2 years [2.1–5.2]; body weight: 6.5 kg [5.4–9.1]) included 4 CKCS, 3 ST, 1 B, 1 D, 1 KC, and 1 MP. All conventional echocardiographic and Doppler variables were within the reference ranges (Gonçalves et al., 2002; Chetboul et al., 2005). Similarly, none of them were hypo- or hypertensive (mean ± SD [ranges] systemic systolic arterial blood pressure: 148 ± 10 mmHg [130–158]) (Brown et al., 2007), and no arrhythmia was detected during the echocardiographic examinations, using concomitant ECG tracing (mean ± SD [ranges] heart rate: 115 ± 18 bpm [90–140]).

The 11 outlier dogs were also rechecked 3 months after the first blood sampling for plasma NT-proBNP concentrations using the same procedure described above. At recheck, the plasma NT-proBNP concentration was 2040 pmol/l [775–2842] *versus* 2842 pmol/l [2842–2842] at the first visit. Only 4/11 dogs still had NT-proBNP concentrations >2842 pmol/L, and those of the 7 remaining dogs

Table 1
Epidemiologic characteristics of the reference sample group including 154 healthy adult small size dogs from 7 different breeds.

Variable	Global	Breed						
		B	CKC	KC	D	MP	ST	YT
n dogs (%)	154	8 (5.2)	36 (23.4)	17 (11.0)	27 (17.5)	20 (13.0)	28 (18.2)	18 (11.7)
n breeders*	28	2	8	4	4	5	5	5
Sex (%)								
F	96 (62.3)	4 (4.2)	21 (21.9)	14 (14.6)	15 (15.6)	12 (12.5)	17 (17.7)	13 (13.5)
M	58 (37.7)	4 (6.9)	15 (25.9)	3 (5.2)	12 (20.7)	8 (13.8)	11 (19.0)	5 (8.6)
Age (years)								
Median	3.2	4.2	2.3	3.7	2.9	3.4	3.4	3.9
IQR	1.9–4.8	2.7–5.5	1.7–3.8	2.4–6.4	1.6–4.2	1.8–4.9	1.9–5.6	2.4–5.9
BW (kg)								
Median	6.5	4.4	8.4	7.5	5.0	5.5	6.5	3.2
IQR	4.6–8.3	3.1–6.8	7.8–8.9	6.6–8.3	4.3–8.9	4.4–6.9	5.7–8.0	2.3–3.3

n: Number of dogs; Global: the tested overall population; F: female; M: male; B: Bichon; CKC: Cavalier King Charles spaniel; D: Dachshund; KC: King Charles spaniel; MP: miniature Poodle; ST: Shih-Tzu; YT: Yorkshire Terrier; BW: body weight; IQR: interquartile range.
* Animals from different breeds could belong to the same breeder.

Table 2
Descriptive statistics for plasma NT-proBNP assessed in 154 healthy adult small size dogs from 7 different breeds.

	Global	Breed						
		B	CKC	KC	D	MP	ST	YT
Number of dogs	154	8	36	17	27	20	28	18
NT-proBNP (pmol/L)								
Median	783	804	1053	964	395	791	868	602
IQR	476–1232	700–1329	791–1446	637–1317	278–566	553–1436	533–1426	465–930

Global: the overall tested population; F: female; M: male; B: Bichon; CKC: Cavalier King Charles spaniel; D: Dachshund; KC: King Charles spaniel; MP: miniature Poodle; ST: Shih-Tzu; YT: Yorkshire Terrier; IQR: interquartile range.

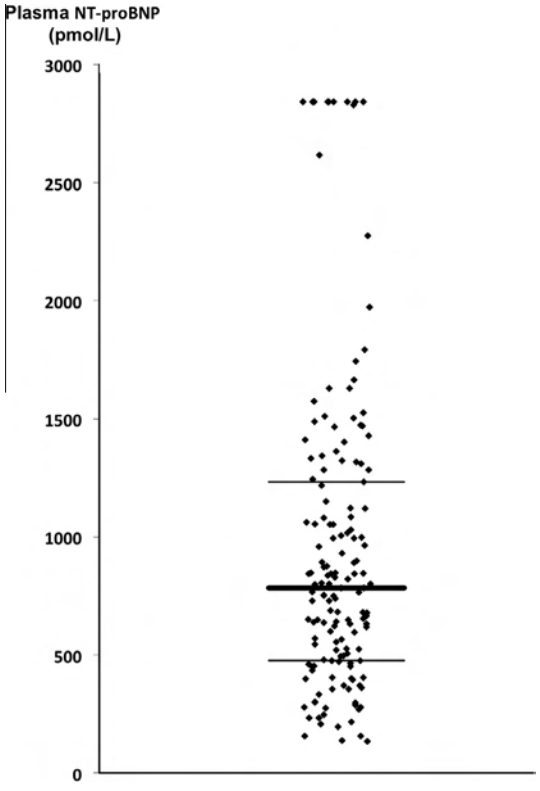

Fig. 1. Distribution of plasma NT-proBNP concentrations among the 154 healthy small size dog population. The thick horizontal bar represents the median value (i.e., 783 pmol/L) and the thin horizontal bars represent the first and third quartiles (i.e., 476 and 1232 pmol/L, respectively).

were between 365 and 2455 pmol/L (Fig. 2). None of the 11 outlier dogs showed an increase in plasma BUN and creatinine, i.e., mean ± SD [ranges] 4.83 ± 1.33 mmol/L [3.33–8.34] and 47.7 ± 10.6 µmol/L [26.5–70.7], respectively (RI provided by the laboratory (Laboratoire Vebiotel, Arcueil, France): 3.33–8.34 mmol/L and 53.0–132.6 µmol/L, for BUN and creatinine, respectively). Owing to these results, none of the 11 outliers were excluded for RI establishment.

3.4. Reference intervals for plasma NT-proBNP

Reference intervals were established in the whole reference sample group (n = 154 dogs). The distribution of plasma NT-proBNP concentration was tested for normality and could not be transformed to fit a Gaussian distribution. The corresponding lower (LL) and upper (UL) limits of the RI with 90% CI were, respectively, 157 [134–233] and 2842 [2842–2842] pmol/L.

4. Discussion

In the present study, plasma NT-proBNP concentration was measured in a large healthy adult small size dog population using standardized procedures with strict inclusion and exclusion criteria, in order to minimize sources of pre-analytical variations. Moreover, RI determination was performed according to the statistical procedures recommended by the CLSI guidelines, to which the American Society for Veterinary Clinical Pathology recently recommended adherence (ASVCP Quality Assurance and Laboratory Standards Committee, 2012). Despite these precautions, the plasma NT-proBNP concentrations exhibited a wide range of values, suggesting inter-individual variability, although all dogs belonged to the same size category. Hence, in the general population, the inter-individual CV was 96% and the within-breed inter-individual CVs ranged from 62% (CKC) to 100% (MP). When considering several other studies (Table 3) on plasma/serum NT-proBNP including groups of healthy dogs (Oyama et al., 2008; Atkinson et al., 2009; Kellihan et al., 2009, 2011; Raffan et al., 2009; Schmidt et al., 2009; Chetboul et al., 2009; Collins et al., 2010; Wess et al., 2011; Cunningham et al., 2012; Hezzell et al., 2012), two reports included healthy comparable breeds as here (i.e., small size dogs), but had a too small sample size (i.e., less than 31, *versus* 154 in the present study) to allow any conclusion on inter-individual variability (Chetboul et al., 2009; Hezzel et al., 2012). In one report including a high number of dogs (n = 196) from the same large breed (i.e., Doberman Pinscher), inter-individual CV according to age category reached 53% in dogs ⩽3 years (Wess et al., 2011). Nevertheless, in the latter studies, the material and methods used for sample handling and assays were different as in our study, which represents a limitation for such comparisons.

Eleven NT-proBNP outliers were detected in the present study. All 11 dogs were confirmed to be free of cardiovascular diseases using a complete standard echocardiography and Doppler examination as well as systemic arterial blood pressure measurement, and were therefore kept for statistical analysis. When they were re-tested for plasma NT-proBNP 3 months later using the same procedures, only 4/11 dogs had plasma NT-proBNP values that still exceeded the upper limit of detection of the assay, and the 7 remaining dogs showed a 1.2 to 6-fold decrease from their initial NT-proBNP value. These findings are in accordance with a study performed on the weekly variability of plasma NT-proBNP in 28 healthy dogs (Kellihan et al., 2009), showing that the difference between maximal and minimal NT-proBNP values was >500 pmol/L, and between 100 and 200 pmol/L in 20% and 40% of the recruited dogs, respectively. In healthy humans, NT-proBNP intra-individual variability may be high, i.e., between 26% and 130% according to studies (Melzi d'Eril et al., 2003; Wu et al., 2003), which thus hampers the interpretation of changes in this biomarker with disease progression and therapy adjustments in patients with cardiovascular diseases (Bruins et al., 2004; Miller et al., 2009). Nevertheless, owing to the very small number of outlier dogs

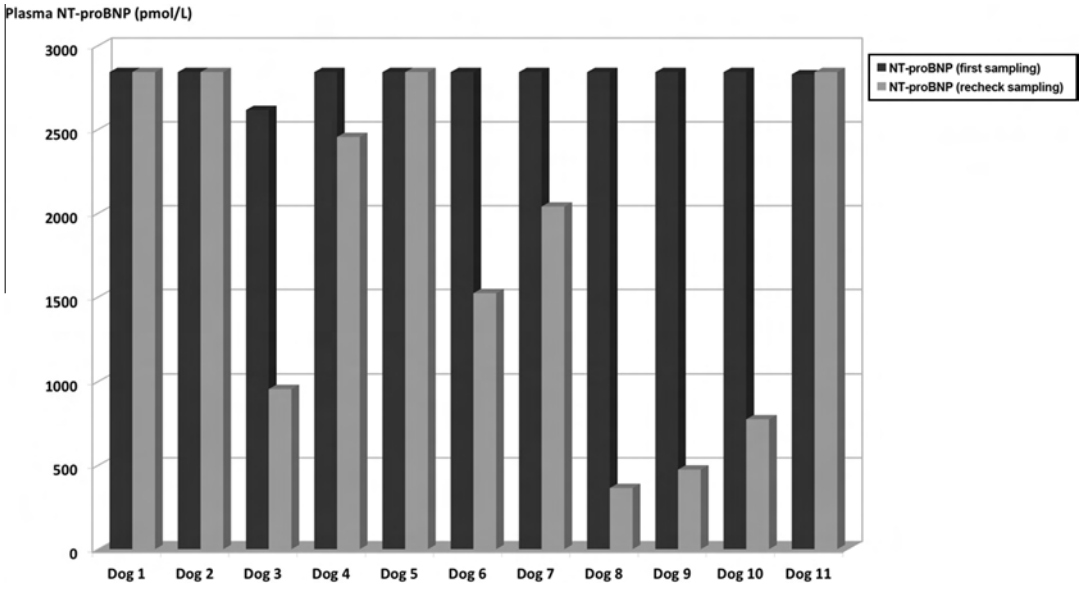

Fig. 2. Comparison between plasma N-terminal pro-B-type natriuretic peptide (NT-proBNP) concentrations assessed in the 11 outlier dogs at first sampling and 3 months later.

Table 3
Comparison of plasma/serum N-terminal pro-B-type natriuretic peptide values in healthy dogs from 11 studies.

Authors	*n*	Age (year)	Body weight (kg)	Breeds	Plasma/serum NT-proBNP (pmol/L)
Cunningham et al. (2012)*	17	Mean ± SD 5.8 ± 2.9	Mean ± SD 30.0 ± 6.9	Mixed breed, Doberman Pinscher, Golden Retriever and other breeds	Median [ranges] 462 [38–1210]
Hezzell et al. (2012)	30	Median (IQR) 9.0 (6–11)	Median (IQR) 11 (7.2–13.8)	Cross-breeds, Cavalier King Charles Spaniel (*n* = 7), Miniature Poodle, Yorkshire Terrier, Border Collie	Median (IQR) 324 (167–530)
Kellihan et al. (2011)	8	Median [ranges] 6 [1–9]	Median [ranges] 28.5 [3.4–55.0]	Great Dane, German shepherd dog, Rottweiler, Dachshund, Boxer, Maltese, Bearded Collie, Cocker spaniel.	Median [ranges] 744 [531–2710]
Wess et al. (2011)	196	Mean 4.4	Mean 34.4	Doberman Pinscher	Median [ranges] 303 [22–1325]
Collins et al. (2010)	10	Data not shown	Data not shown	Cavalier King Charles Spaniels, crossbreds, Boxers, Lurchers, English Springer and Cocker Spaniels, Doberman Pinscher, Bull Terriers, Labrador and Golden Retriever, Great Dane, Irish, Wolfhound, Gordon and Irish Setter, Whippet, Miniature Schnauzer, Fox Terrier	Median [ranges] 413 [356–487] before freezing 709 [378–825] after freezing
Atkinson et al. (2009)	9	Median 5	Median 19.8	Data not shown	Median [ranges] 373 [209–738]
Chetboul et al. (2009)	22	Mean ± SD 9.1 ± 1.6	Mean ± SD 6.8 ± 3.6	Small size breeds: King Charles and Cavalier King Charles Spaniel, Bichon, Yorkshire Terrier (*n* = 12); Other small breeds (*n* = 10)	Median [ranges] 278 [68–515]
Kellihan et al. (2009)	28	Median 7	Median 20.4	Mixed breed, Labrador and Golden Retrievers, American Staffordshire Terriers, other breeds	Median [ranges] Week 1: 377 [159–631] Week 2: 320 [159–632] Week 3: 358 [159–650]
Raffan et al. (2009)	39	Mean 6.0	Data not shown	Unknown	Median [ranges] 118 [2–673]
Schmidt et al. (2009)	23	Median (IQR) 7 (5–8)	Median (IQR) 17.7 (10–30)	Mixed breed (48%), Golden Retriever, Keeshonds and other breeds	Mean (95%CI) 261 (225–303)
Oyama et al. (2008)	40	Median (IQR) 7.0 (4.3–8.0)	Median (IQR) 19.9 (8.5–30.5)	Mixed breed (40%), Great Dane, Golden retriever and other breeds	Median (IQR) 290 (478–598)

n: number of dogs; IQR: interquartile range; CI: confidence interval; SD: standard deviation.
* Protocol using protease tubes.

(*n* = 11), intra-individual variability of plasma NT-proBNP could not be assessed in the present study.

As described in humans (Loke et al., 2003) and in one recent report in dogs (Wolf et al., 2013), a sex effect on plasma NT-proBNP was observed in the present study, with females having higher median plasma NT-proBNP values than males. Conversely, no age or body weight effects were found, in accordance with several other reports (Boswood et al., 2008; Tarnow et al., 2009; Kellihan et al., 2009; Oyama et al., 2008; Ettinger et al., 2012). However plasma NT-proBNP concentration has been shown to be

significantly increased in healthy Doberman Pinschers >8 years as compared with younger dogs of the same breed (Wess et al., 2011), and to be inversely related to body weight in a group of 39 asymptomatic CKCS with DMVD (Tarnow et al., 2009). Nevertheless, the absence of a significant effect of age on plasma NT-proBNP may be attributable to the fact that all recruited dogs were < 10 years old, and this could represent a limitation of the present study.

Two studies reported a significant breed effect for plasma NT-proBNP (Oyama et al., 2008; Häggström et al., 2012). However, no breed effect was observed in the present study. This may be explained by the fact that all dogs belonged to the same size category.

This study presents several limitations. Firstly, plasma NT-proBNP concentrations were only measured on a second occasion in dogs that were found to have exceptionally high concentrations at the first time of measurement. Since these dogs were outliers, it is likely that when measured on a second occasion, the variability in their concentrations would be greater than that seen by more typical members of the population (Bland and Altman, 1994a,b). Therefore, conclusions cannot be drawn about natural variability of plasma NT-proBNP concentration in typical individual dogs from this study. Another limitation is that the UL of the RI determined in the present study corresponds to the limit of quantification, as the commercial laboratory responsible for the NT-proBNP assays did not assess plasma NT-proBNP values >2842 pmol/L. Therefore, the UL of the RI should be interpreted with caution. Moreover, complementary cardiovascular examinations were only scheduled for the outliers, and not for all the recruited dogs. However, one important step, as for any study of reference intervals, was to define the criteria for health. Since echocardiography is not currently considered as a prerequisite for NT-proBNP testing, the present study was designed so that the conditions were similar to those encountered in routine clinical practice, and dogs were considered healthy on the basis of a complete physical examination, history and routine plasma biochemistry. Finally, effect of sample handling and storage could have affected plasma NT-proBNP concentrations, as serum NT-proBNP has been shown to decrease at room temperature and increase after storage at −20 °C (Collins et al., 2010). Nevertheless, according to the latter results, the authors recommend that samples should be separated and frozen within 1 h of blood collection and sent frozen to the laboratory until assays, and this was the case in the present study.

In conclusion, this study suggests that plasma NT-proBNP concentration is characterized by a high inter-individual variability in healthy adult small size dogs. Therefore, a single plasma value in a healthy small size dog should be interpreted with caution. Moreover, a sex effect has been demonstrated, with females having higher concentrations than males, but the clinical relevance of partitioning the RI according to sex needs further investigations. Prospective studies including a larger sample size and other breeds are therefore needed to better understand NT-proBNP physiology in dogs.

Conflict of interest

The Vivid i ultrasound system used in the study was lent by Scil Animal Care Company (67120 Altorf, France). Novartis Animal Health supported C. Misbach's PhD program.

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Journal of Veterinary Cardiology (2014) **16**, 91—100

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Journal of Veterinary Cardiology ESVC

www.elsevier.com/locate/jvc

Echocardiography and conventional Doppler examination in clinically healthy adult Cavalier King Charles Spaniels: Effect of body weight, age, and gender, and establishment of reference intervals

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Received 25 October 2013; received in revised form 16 March 2014; accepted 17 March 2014

KEYWORDS
Canine;
Cardiac;
Dog;
Heart;

Abstract *Objectives:* The objectives of this study were (1) to assess the potential effect of body weight (BW), age, and gender on the most commonly used echocardiographic and conventional Doppler variables in a large population of healthy Cavalier King Charles Spaniels (CKCS), and (2) to establish the corresponding reference intervals (RI).

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1760-2734/$ - see front matter
http://dx.doi.org/10.1016/j.jvc.2014.03.001

Ultrasound

Animals: 134 healthy adult CKCS.
Methods: Ultrasound examinations were performed by trained observers in awake dogs. M-mode variables included left ventricular (LV) end-diastolic and end-systolic diameters, LV free wall and interventricular septal thicknesses at end-diastole and end-systole, and LV fractional shortening (FS%). The left atrium (LA) and aortic (Ao) diameters were measured using a 2D method, and the LA/Ao was calculated. Pulsed-wave Doppler variables included peak systolic aortic and pulmonary flow velocities, mitral E and A waves, and E/A ratio. Effects of BW, age, and gender on these 15 variables were tested using a general linear model, and RIs were determined by applying the statistical procedures recommended by the Clinical and Laboratory Standards Institute.
Results: A significant BW effect was observed for all variables, except LA/Ao, FS%, and mitral E/A ratio. A significant but negligible effect of gender and age was also observed for 5/15 and 4/15 of the tested variables, respectively. Only the BW effect on M-mode variables was considered as clinically relevant and the corresponding regression-based RIs were calculated.
Conclusions: Body weight should be taken into account when interpreting echocardiographic values in CKCS, except for LA/Ao, FS%, and mitral E/A ratio.

The Cavalier King Charles Spaniel (CKCS) breed is known to be highly predisposed to develop degenerative mitral valve disease (DMVD), with a prevalence that may attain 100% in geriatric populations.[1–5] Based on the classification system proposed by the American College of Veterinary Internal Medicine (ACVIM) Consensus Statement on DMVD and including 4 stages (A to D),[6] all CKCS (even those without a heart murmur) can be assigned to stage A (i.e., are considered at high risk for developing the disease). Therefore, according to the ACVIM consensus recommendations, CKCS should undergo yearly cardiac screening as part of their regular health evaluations.[6] Standard transthoracic echocardiography is currently considered as the non-invasive diagnostic method of choice for early detection of DMVD-associated mitral valve lesions, evaluation of mitral regurgitation severity, and for assessing its impact on myocardial function and cardiac remodeling (left atrial and ventricular dilation).[7] However, for such a purpose, the measured echocardiographic values need to be compared with reference intervals (RI) established in healthy dogs. Reference intervals are crucial for accurate identification of the morphological and functional cardiac changes associated with the disease. The concept of RI is based on determining a set of values within which 95% of the values of a particular variable in a population would fall.[8] No specific recommendations for the determination of RI are available in veterinary cardiology. The American Society of Veterinary Clinical Pathology advises adherence to the Clinical Laboratory and Standard Institute (CLSI) recommendations[9] and has provided guidelines for the determination of *de novo* RI in veterinary species.[10]

Due to the large variations in size and conformation within the canine population, significant breed and body weight (BW) effects on echocardiographic variables have been demonstrated,

List of abbreviations

2D	two-dimensional
ACVIM	American College of Veterinary Internal Medicine
Ao	aortic
BW	body weight
CI	confidence intervals
CKCS	Cavalier King Charles spaniel
CLSI	Clinical Laboratory and Standard Institute
DMVD	degenerative mitral valve disease
FS%	fractional shortening
IVSd	interventricular septum thickness at end-diastole
IVSs	interventricular septum thickness at end-systole
LA	left atrium
LV	left ventricular
LVDd	left ventricular end-diastolic diameter
LVDs	left ventricular end-systolic diameter
LVFWd	left ventricular free wall thickness at end-diastole
LVFWs	left ventricular free wall thickness at end-systole
RI	reference intervals

mainly for left ventricular (LV) M-mode measurements.[11–13] Body weight independent differences among breeds have also been reported.[11] For these reasons, the breed must be considered when establishing echocardiographic RI in the dog.[11] Several methods have been proposed, either for the overall canine population[14–20] or in specific breeds and breed categories.[21–34] Some studies also demonstrated an age and/or gender effect on echocardiographic and Doppler variables, but the need to develop RI according to these covariates remains unclear.[22,24–26,28,31,33,34] However, to the best of our knowledge, no study has focused on the determination of RI for echocardiographic and conventional Doppler variables in the CKCS breed.

The aims of this study were therefore (1) to assess the potential effect of BW, age, and gender on the most commonly used standard echocardiographic and conventional Doppler variables in a large population of clinically healthy adult CKCS, and (2) to establish the corresponding RI according to the statistical procedures recommended in the CLSI guidelines.[9]

Animals, material and methods

Animals

The case records of breeder- and client-owned healthy adult (≥12 months) CKCS that prospectively underwent a cardiac screening using a standardized protocol at the Cardiology Unit of Alfort (National Veterinary School of Alfort, France) between March 2001 and January 2013 were reviewed. Dogs were considered healthy on the basis of a complete physical examination. Dogs with abnormal results in the physical examination and those on medication were excluded from the study.

Standard echocardiography and conventional Doppler examinations

Standard echocardiography and conventional Doppler examinations were performed by trained observers, with at least 3 years of experience in cardiology, in awake dogs gently restrained in standing position, using continuous ECG monitoring with an ultrasound unit[e] equipped with 3S (1.5–3.5 MHz), 5S (2.2–5 MHz), and 7S (3.5–8 MHz) phased-array transducers, as previously described and validated.[35] Left ventricular measurements were obtained using two-dimensional (2D) guided M-mode as recommended by the American Society of Echocardiography,[36] from the right parasternal transventricular short-axis view. Measurements were made of the LV end-diastolic (LVDd) and end-systolic (LVDs) diameters as well as LV free wall and interventricular septum thicknesses at end-diastole (LVFWd and IVSd) and end-systole (LVFWs and IVSs). For each M-mode echocardiographic variable, a mean of 3 measurements was determined from 3 consecutive cardiac cycles on the same frame. The LV fractional shortening (FS%) was then calculated. Measurements of the aortic (Ao) and left atrium (LA) diameters were obtained at end-diastole with a 2D method from the right parasternal transaortic short axis view and using the calipers position described by Hansson et al.[35,37] The LA/Ao ratio was then calculated.

The maximal systolic pulmonary and aortic flow velocities were determined by pulsed-wave Doppler using the right parasternal transaortic short axis and left apical five-chamber views, respectively. Early and late diastolic mitral inflow velocities (mitral E and A waves) were also measured from the left apical four-chamber view, and the E to A ratio was calculated. Finally, morphology of the mitral valve apparatus was assessed and color-flow Doppler examination was performed using right and left apical four-chamber views in order to exclude DMVD (i.e., thickening of mitral valve leaflets and *chordae tendineae* and/or mitral valve prolapse associated with mitral regurgitation). All other cardiac valves were also assessed using both 2D and color-flow Doppler modes. Mean heart rate was calculated by ECG monitoring from the same cardiac cycles used for the M-mode measurements.

Statistical analysis

Data were expressed as median and range. Outliers were identified and RI determined by applying the statistical procedures recommended by the CLSI guidelines.[9] Native and Box–Cox transformed data were first tested for normality by use of the Anderson-Darling test. Outliers were identified by applying the Tukey method. When the data distribution remained non-Gaussian after Box–Cox transformation, the values in both tails of the distribution were subjected to visual inspection.

Effects of covariates (BW, age, and gender) on standard echocardiographic and conventional

[e] Vingmed system 5, Vivid 5, Vivid 7 dimension and Vivid 7 BT03, General Electric Medical System, Waukesha, WI, USA.

Table 1 Median and ranges (minimum–maximum) of echocardiographic and Doppler variables in the whole study population and according to gender (males and females).

	All	Males	Females
Number of animals	134	62	72
Heart rate (beats/min)	120 (65–170)	120 (65–160)	120 (80–170)
Dimensional variables			
LA (mm)	13.4 (9.7–19.7)	14.1 (11.0–19.7)	12.9 (9.7–17.8)
Ao (mm)	18.3 (14.3–23.9)	18.9 (15.6–23.4)	18.1 (14.3–23.9)
LA/Ao	0.74 (0.47–0.94)	0.76 (0.47–0.93)	0.72 (0.51–0.94)
LVDd (mm)	29.1 (21.4–41.1)	29.8 (23.3–41.1)	28.6 (21.4–34.7)
LVDs (mm)	17.6 (10.9–24.6)	18.8 (13.1–24.6)	16.8 (10.9–21.0)
LVFWd (mm)	6.4 (4.9–8.5)	6.4 (5.2–8.5)	6.3 (4.9–8.4)
LVFWs (mm)	10.8 (7.8–14.8)	10.9 (8.2–14.0)	10.6 (7.8–14.8)
IVSd (mm)	6.5 (4.8–8.8)	6.7 (4.8–8.8)	6.2 (4.8–8.2)
IVSs (mm)	10.1 (6.9–14.1)	10.4 (6.9–14.1)	10.0 (7.0–14.0)
Systolic function variables			
FS (%)	39.1 (30.7–51.9)	38.3 (30.7–40.1)	41.1 (31.0–51.9)
Vmax Ao (m/s)	1.14 (0.75–1.71)	1.14 (0.80–1.71)	1.14 (0.75–1.70)
Vmax PA (m/s)	0.80 (0.50–1.56)	0.81 (0.50–1.56)	0.80 (0.55–1.10)
Diastolic function variables			
E (m/s)*	0.72 (0.53–1.03)	0.76 (0.54–0.99)	0.72 (0.53–1.03)
A (m/s)*	0.50 (0.28–0.79)	0.50 (0.28–0.77)	0.50 (0.29–0.79)
E/A*	1.4 (1.5–2.4)	1.4 (1.1–2.4)	1.4 (1.0–2.2)

*Data available from 101 dogs (47 males and 54 females). LA: left atrium, Ao: Aorta, LA/Ao: left atrium/aorta ratio, LVDd and LVDs: end-diastolic and end-systolic left ventricular diameters, respectively, LVFWd and LVFWs: end-diastolic and end-systolic left ventricular free wall thicknesses, respectively, IVSd and IVSs: end-diastolic and end-systolic interventricular septum thicknesses, respectively, FS: fractional shortening, Vmax Ao and Vmax PA: systolic maximal aortic and pulmonary flow velocities, respectively, E and A: mitral E and A wave velocities, respectively, E/A: mitral E/A ratio.

Doppler variables were tested using a statistical software package[f] and the following linear mixed effect model:

$$Y = \mu + \text{Sex} + a\text{Age} + b\text{BW} + \varepsilon,$$

where Y is the value of the echocardiographic variable, μ is a constant term, Age and BW are continuous variables, a and b are the slope coefficients for Age and BW, and ε is the residual term of the model.

Reference intervals were defined as central 95% intervals bounded by the 2.5th and 97.5th percentiles. The upper and lower limits of the RI with their 90% confidence intervals (CI) were determined in the global population by using a non-parametric approach.[38] When a significant effect of a covariate on imaging variables was found, the corresponding regression-based RIs were built.[38,39] A value of $P < 0.05$ was considered significant.

[f] Systat version 8.0, SPSS Inc., Chicago, IL, USA.

Table 2 Slope coefficients for the continuous variables (age and body weight) and differential effects for the categorical variable (gender) in the model used for statistical analysis of echocardiographic and Doppler variables assessed in the whole study population.

	μ	Body weight		Age		Gender	
		Slope coefficient	*P* value	Slope coefficient	*P* value	Differential effect	*P* value
Heart rate (beats/min)	107	–	0.109	–	0.134	–	0.695
Dimensional variables							
LA (mm)	10.88	+0.325	0.003	–	0.526	F: −0.395 M: +0.395	0.018
Ao (mm)	14.02	+0.438	<0.0001	+0.175	0.008	–	0.100
LA/Ao	0.76	–	0.95	–	0.053	–	0.252
LVDd (mm)	20.52	+0.987	<0.0001	–	0.952	F: −0.641 M: +0.641	0.002
LVDs (mm)	13.66	+0.493	<0.0001	–	0.297	F: −0.698 M: +0.698	0.001
LVFWd (mm)	4.43	+0.197	<0.0001	+0.064	0.040	–	0.463
LVFWs (mm)	6.78	+0.390	<0.0001	+0.142	0.003	–	0.351
IVSd (mm)	5.55	+0.102	0.032	–	0.383	F: −0.178 M: +0.178	0.016
IVSs (mm)	7.31	+0.244	0.002	+0.171	0.003	–	0.233
Systolic function variables							
FS (%)	34.8	–	0.153	–	0.187	F: +1.168 M: −1.168	0.012
Vmax Ao (m/s)	0.78	+0.039	0.001	–	0.361	–	0.471
Vmax PA (m/s)	0.61	+0.026	0.003	–	0.290	–	0.725
Diastolic function variables							
E (m/s)*	0.59	+0.017	0.02	–	0.792	–	0.998
A (m/s)*	0.35	+0.015	0.033	–	0.223	–	0.938
E/A*	1.58	–	0.975	–	0.08	–	0.974

*Data available from 101 dogs (47 males and 54 females). M: male, F: female, See Table 1 for remainder of key. The slope coefficients and differential effects are only given when the effect is statistically significant. For example, the general regression equation for interventricular end-diastolic septum (IVSd) in a female CKCS will be: IVSd (mm) = 5.55 − 0.178 + 0.102 × body weight (kg).

Results

A total of 134 healthy adult CKCS, 72 females and 62 males (age: 3.0 years [1.0—12.1]; BW: 8.7 kg [6.0—14.5]), were included in the study. As inclusion criteria, none of the 134 recruited dogs had mitral valve lesions. Similarly, morphology of the aortic, tricuspid, and pulmonary valves were considered normal. Physiologic minor tricuspid and/or pulmonary regurgitations were detected in 94 out of the 134 (70%) recruited dogs using color-flow Doppler mode, whereas no aortic regurgitation was found.

Heart rates and results for the 10 standard echocardiographic and 5 conventional Doppler variables in the whole study population and according to gender are provided in Table 1.

As shown in Table 2, a statistically significant effect of BW was observed for all variables except FS%, LA/Ao, E/A, and heart rate. A significant effect of gender and age was also observed for 5/15 and 4/15 of the tested variables, respectively.

Eight of the 15 distributions of echocardiographic variables tested for normality were Gaussian. Box—Cox transformation yielded a Gaussian distribution for the 2/7 remaining variables. Application of the Tukey method revealed 3 outlier dogs for the maximal systolic pulmonary flow velocity variable, (i.e., values of 1.33, 1.37 and 1.56 m/s). Since echocardiography did not reveal any abnormalities, these outliers were not excluded from the RI determination. The corresponding lower and upper limits of RI for the 15 standard echocardiographic and conventional Doppler variables tested, with 90% CI, are provided in Table 3. Since only the BW effect on M-mode variables was considered as clinically relevant (see Discussion Section), regression-based RIs were built (Table 4). Finally, the M-mode echocardiographic reference intervals (2.5th and 97.5th percentiles) established in the present study according to the

Table 3 Reference intervals (lower and upper limits with 90% confidence intervals) for the 15 standard echocardiographic and conventional Doppler variables assessed in healthy adult Cavalier King Charles Spaniels ($n = 134$) and the corresponding within-day and between-day standard deviations obtained by a trained observer in a previous study.[35]

	Lower limit (90% confidence interval)	Upper limit (90% confidence interval)	Within-day SD	Between-day SD
Dimensional variables				
LA (mm)	10.1 (9.7–10.5)	17.6 (16.7–19.7)	–	–
Ao (mm)	15.5 (14.3–15.9)	21.9 (21.5–23.9)	–	–
LA/Ao	0.54 (0.47–0.56)	0.93 (0.90–0.94)	0.07	0.08
LVDd (mm)	23.4 (21.4–25.3)	35.6 (34.2–41.1)	1.74	2.51
LVDs (mm)	12.9 (10.9–13.8)	23.6 (21.4–24.6)	1.39	1.39
LVFWd (mm)	5.0 (4.9–5.3)	8.3 (7.9–8.5)	0.75	0.64
LVFWs (mm)	8.2 (7.8–8.8)	13.8 (13.2–14.8)	1.02	1.34
IVSd (mm)	5.1 (4.8–5.2)	8.3 (8.0–8.8)	0.84	0.34
IVSs (mm)	7.6 (6.9–8.1)	13.0 (12.5–14.1)	0.90	0.48
Systolic function variables				
FS (%)	31 (30.7–31.6)	50.8 (49.1–51.9)	3.73	5.76
Vmax Ao (m/s)	0.80 (0.75–0.88)	1.62 (1.55–1.71)	–	–
Vmax PA (m/s)	0.55 (0.50–0.61)	1.24 (1.06–1.56)	–	–
Diastolic function variables				
E (m/s)*	0.55 (0.53–0.57)	1.0 (0.93–1.03)	–	–
A (m/s)*	0.32 (0.28–0.35)	0.76 (0.73–0.79)	–	–
E/A*	1.04 (1.01–1.09)	2.17 (2.06–2.37)	–	–

*Values calculated from 101 dogs (47 males and 54 females). SD: standard deviation. See Table 1 for remainder of key.

CLSI guidelines[9] and the predictive reference intervals calculated in the same population using Cornell's formula[20] ($a \times$ body weight^b, with a and b as covariates) are presented in Table 5.

Discussion

Standard 2D and M-mode echocardiography combined with conventional Doppler examination plays a critical role in the initial and longitudinal assessment of dogs affected by DMVD, providing information on mitral valve anatomy, mitral regurgitation severity and its hemodynamic consequences on left heart size and function, as well as cardiac and vascular pressures.[7] The well-known predisposition of the CKCS breed to DMVD[1–5] led us to determine specific RI for the most commonly used standard echocardiographic and conventional Doppler variables in a population of healthy adult CKCS. The major strengths of this study were as follows: (1) the reference sample group was quite large (i.e., $n = 134$ healthy adult CKCS), with a balanced male/female ratio (ratio = 0.86), and a relatively wide range of age (1.0–12.1 years) and BW (6.0–14.5 kg); (2) the RI were determined according to the statistical procedures recommended in the CLSI guidelines[9]; and (3) the imaging variables tested here are of clinical interest, especially with regard to the

Table 4 Predicted regression-based reference intervals (lower and upper limits) according to body weight for the 6 M-mode variables with a significant body weight effect assessed in a population of healthy adult Cavalier King Charles Spaniels ($n = 134$).

Body weight (kg)	LVDd (mm)	LVDs (mm)	LVFWd (mm)	LVFWs (mm)	IVSd (mm)	IVSs (mm)
5	20.2—29.8	10.6—20.3	4.0—7.0	6.6—11.3	4.3—7.7	6.0—11.6
6	21.3—30.9	11.2—20.8	4.2—7.3	7.1—11.7	4.5—7.8	6.4—11.9
7	22.5—31.9	11.8—21.3	4.5—7.5	7.6—12.2	4.6—7.9	6.7—12.2
8	23.6—33.0	12.3—21.9	4.7—7.7	8.1—12.6	4.8—8.1	7.1—12.5
9	24.6—34.1	12.9—22.4	4.9—7.9	8.5—13.1	4.9—8.2	7.4—12.9
10	25.8—35.2	13.5—23.0	5.2—8.1	9.0—13.5	5.1—8.3	7.7—13.2
11	26.8—36.3	14.0—23.6	5.4—8.4	9.4—14.0	5.2—8.5	8.0—13.5
12	27.8—37.4	14.5—24.1	5.6—8.6	9.9—14.5	5.3—8.6	8.3—13.9
13	28.8—38.5	15.0—24.7	5.8—8.8	10.3—14.9	5.4—8.8	8.6—14.2

kg: kilograms, See Table 1 for remainder of key.

Table 5 M-mode echocardiographic reference intervals (2.5th and 97.5th percentiles) from 134 healthy adult Cavalier King Charles Spaniels determined according to the Clinical and Laboratory Standards Institute (CLSI) statistical procedures (A),[9] and predictive reference intervals calculated in the same population using Cornell's formula (B).[20]

M-mode echocardiographic variable	A	B
LVDd (mm)	23.4—35.6	21.5—40.6
LVDs (mm)	12.9—23.6	12.0—27.7
LVFWd (mm)	5.0—8.3	4.9—13.2
LVFWs (mm)	8.2—13.8	8.1—19.1
IVSd (mm)	5.1—8.3	4.9—13.0
IVSs (mm)	7.6—13.0	7.3—17.3

See Table 1 for remainder of key.

pathophysiology of DMVD, which can potentially lead to LA and LV dilation as well as myocardial dysfunction. Additionally, some of the tested echo-Doppler variables have been shown to be related to clinical outcome in DMVD dogs. For example, the high prognostic value of the degree of LA dilation, as assessed by the LA/Ao ratio, has been demonstrated in both symptomatic and asymptomatic dogs with DMVD.[7,40—45] Similarly, a high velocity mitral E wave has been shown to be associated with a higher risk of death or decompensation in dogs with DMVD.[41,42,45]

In the present study, although all the M-mode echocardiographic RI established according to the CLSI guidelines were within the predictive RI calculated using Cornell's formula,[20] all were more narrow compared to the latter, as illustrated by the end-diastolic LVFW (i.e., 5.0—8.3 mm in the present study *versus* 4.9—13.2 mm with Cornell's formula). In accordance with Morrison et al.,[11] these results suggest that not only the BW but also the somatotype should be considered when establishing echocardiographic measurement RI in the dog.

Another objective of the present study was to assess the effect of covariates (i.e., BW, age, and gender) on the standard echocardiographic and conventional Doppler variables tested. A significant BW effect was observed for all variables except the 3 M-mode, 2D, and Doppler ratios, i.e., FS%, LA/Ao, and E/A respectively, the latter 2 being the sole variables not significantly affected by any of the tested covariates. Unlike these ratios, all imaging variables ($n = 12$) were significantly affected by BW as well as gender (4/12) and/or age (4/12). These results are in accordance with most echocardiographic studies which focussed on specific breeds and included the CKCS breed, and showed that BW is significantly correlated with most M-mode LV measurements, but not with LA/Ao.[22,24—26,29,34] Nevertheless, the clinical relevance of these results needs to be discussed with regard to the observers' variability.[35] Using the general linear model (see Table 2), if BW is increased by 4 kg, the IVS and LVFW thicknesses will be increased by 0.41 and 0.79 mm in diastole, and by 0.98 and 1.56 mm in systole, respectively. These differences are higher than

the between-day standard deviations assessed by our group for a trained observer (i.e., 0.34 mm, 0.64 mm, 0.48 mm, and 1.34 mm, respectively),[35] and therefore can be considered as clinically relevant. Similarly, for a 4 kg BW increase, the LVDd and LVDs will be increased by 3.95 mm and 1.97 mm, respectively (with corresponding between-day standard deviations of 2.51 mm and 1.39 mm, respectively). Conversely, the statistically significant effect of gender on LVDd, LVDs, and IVSd can be considered as clinically negligible, as demonstrated by the low slope coefficients (0.641, 0.698, 0.178) in comparison to the corresponding between-day standard deviations (2.51, 1.39, 0.34). A similar rationale can be applied to the statistically significant age effect on the 3 M-mode variables, i.e., LVFWd, LVFWs, and IVSs. For example, when age is increased by 8 years, the LVFWd and LVFWs will be increased by less than the corresponding between-day standard deviations, except for IVSs, which is nevertheless not considered as the most relevant variable for assessing canine DMVD. Lastly, no significant effect of covariates on the 4 Doppler variables was found, except for BW. Nevertheless, this BW effect can also be considered negligible, as a 4 kg BW increase will only increase peak velocities from 0.06 m/s to 0.15 m/s. It has been recently advocated that the decision to partition an RI into subclasses according to covariates should not only be made on the basis of statistical concepts, but also if physiologic differences are expected to result in important clinical differences in RI.[46] According to the results of the present study, it can therefore be assumed that only BW should be taken into account when interpreting M-mode echocardiograms in the CKCS breed. Therefore, the use of regression-based RIs according to BW were proposed for the 6 M-mode variables.[47]

This study presents several limitations. Firstly, the present study allowed determination of 'reference limits' (i.e., the 2.5th or 97.5th percentiles bounding the RI) and the corresponding 90% CI in a healthy population of CKCS. The 'decision limits', that is to say predetermined thresholds that help distinguish 2 populations, should be further defined by consensus and based on investigations on individuals with and without a specific disease or conditions. Moreover, an RI should be interpreted in light of each observer's variability, which should ideally be assessed for the considered breed, which was not the case in the present study. Another limitation is that the study population was relatively young (i.e., median age of 3 years), as most recruited CKCS were breeder-owned dogs that underwent cardiac screening before mating. For this reason, the effect of age on the tested variables could have been underestimated, especially for mitral flow velocities, which have been demonstrated to be modified with age, particularly in dogs >6 years old.[48] On the other hand, screening for cardiac diseases in CKCS is often performed in young adult dogs.[1,49] Another limitation is that the body condition score of the 134 recruited dogs was not assessed, and not only BW *per se*, but also body size may have influenced some of the present results. Moreover, the LA and Ao diameters were both assessed from 2D frames at a specific phase of the cardiac cycle, i.e., at end-diastole. Therefore, and as for the 15 variables assessed in the present study, the corresponding RI cannot not be applied to measurements obtained using another methodology (i.e., at another time of the cardiac cycle, from another view or using another mode). A further limitation is that the E and A mitral flow velocities and the E/A ratio were only recorded in 101/134 dogs, because cardiac screening protocols for CKCS only began to systematically include these Doppler variables in 2006. The CLSI guidelines recommend that when using the non-parametric approach to determine RI, the sample size should ideally be > 120 subjects, which was not the case for these 3 variables.

In conclusion, although predictive RI assessed according to Cornell's formula seem to be acceptable for CKCS dogs, the present results suggest that establishing specific RI for echocardiographic and Doppler variables in this breed is relevant. The present report provides RI for standard echocardiographic and conventional Doppler variables, and demonstrates a statistically significant and clinically relevant BW effect on M-mode echocardiographic variables including end-diastolic and end-systolic LV wall thicknesses and diameters. Body weight should therefore be considered when interpreting echocardiographic values in CKCS, except for the 2 2D and M-mode ratios, i.e., LA/Ao and FS% respectively. Further prospective investigations in larger populations, with wider ranges of ages, and including more variables of interest (i.e., LV volumes, right chambers dimensions) are needed to better determine ultrasound-derived cardiovascular imaging RIs within the CKCS breed.

Conflict of interest

None.

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Misbach Charlotte

Determination of reference intervals in small size dogs for variables used in veterinary cardiology

Summary: Degenerative mitral valve disease is the most common heart disease in small size dogs. Several echocardiographic, Doppler and blood variables are crucial in the assessment of the disease but need to be interpreted in the light of a specific reference interval (RI). The aim of this work was to determine RI for 31 variables of clinical interest in veterinary cardiology within a large population of healthy small size dogs by using the *Clinical and Laboratory Standard Institute* recommendations. The three studies performed here allowed to conclude that determination of specific RI in this canine sub-population is relevant. Moreover, the effect of covariates such as body-weight, age and gender should be taken into account only if a clinical interest is identified.

Key words: biochemistry, biomarker, canine, cardiac ultrasound, heart, population, reference value

Misbach Charlotte

Détermination d'intervalles de référence chez les chiens de petit format pour des variables d'utilité en cardiologie vétérinaire

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Thèse soutenue le mardi 24 février 2015 à l'Ecole Nationale Vétérinaire de Toulouse

Résumé : La dégénérescence valvulaire mitrale (MVD) est la cardiopathie la plus fréquente chez le chien de petit format. Certaines variables écho-Doppler et sanguines sont incontournables dans son évaluation mais nécessitent d'être interprétées selon un intervalle de référence (IR) spécifique. L'objectif de ce travail a été de déterminer des IR pour 31 variables d'utilité clinique en cardiologie vétérinaire dans une population importante de chiens sains de petit format et selon les recommandations du *Clinical and Laboratory Standard Institute*. Les trois études réalisées permettent de conclure que l'élaboration d'IR spécifiques dans une sous-population canine est pertinente pour certaines variables. De plus, l'effet de certains facteurs comme le poids, l'âge et le sexe doivent être pris en compte si un intérêt clinique est identifié.

Mots clés : biochimie, biomarqueur, canidé, cœur, imagerie ultrasonore, population, valeur de référence

Discipline : Pathologie, Toxicologie, Génétique et Nutrition

Unités de Recherche : Unité de Recherche Clinique, Département des Sciences Cliniques, Ecole Nationale Vétérinaire de Toulouse et Unité de Cardiologie d'Alfort, Centre Hospitalier Universitaire Vétérinaire d'Alfort, Ecole Nationale Vétérinaire d'Alfort.