



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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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.

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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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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 sampled 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, 1500g) (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 $\pm$ 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 pulmonary 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 ≥ 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 $\pm$ 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 $\pm$ 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
<i>n</i> dogs (%)	154	8 (5.2)	36 (23.4)	17 (11.0)	27 (17.5)	20 (13.0)	28 (18.2)	18 (11.7)
<i>n</i> 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.

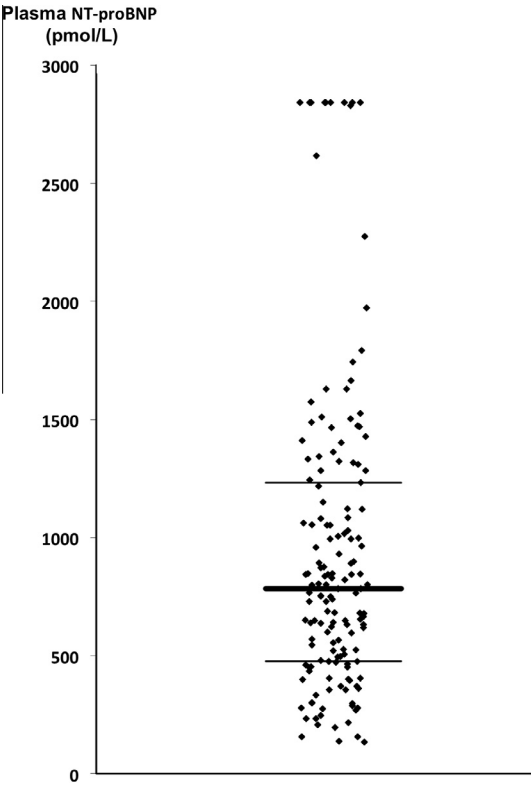


Fig. 1. Distribution of plasma NT-proBNP concentrations among the 154 healthy small size dog population. The thick horizontal bar represents the median value (i.e., 783 pmol/L) and the thin horizontal bars represent the first and third quartiles (i.e., 476 and 1232 pmol/L, respectively).

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 $\pm$ SD [ranges] 4.83 ± 1.33 mmol/L [3.33–8.34] and 47.7 ± 10.6 μ mol/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 μ mol/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; Hezzell 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

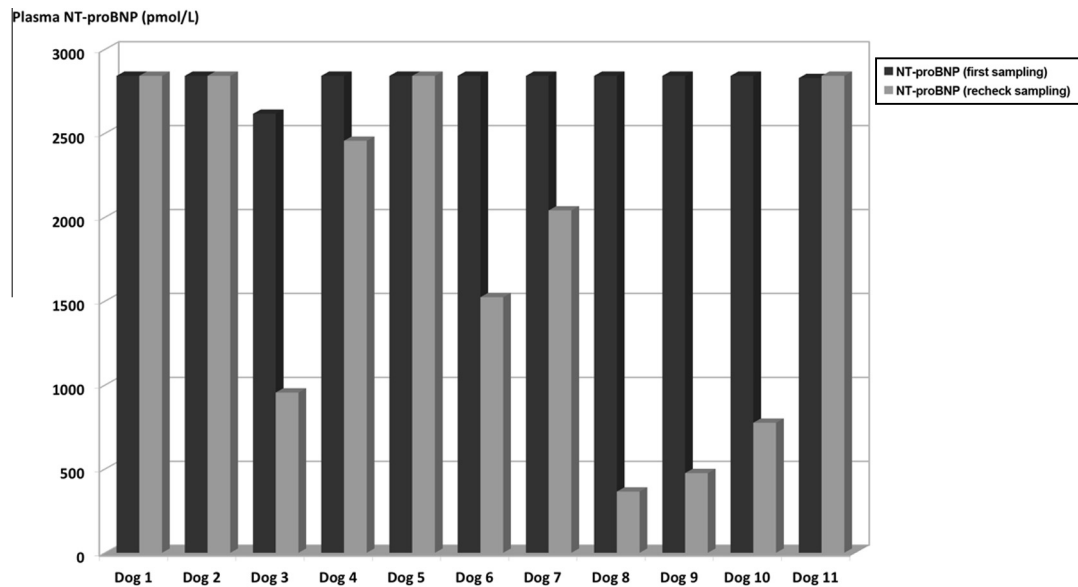


Fig. 2. Comparison between plasma N-terminal pro-B-type natriuretic peptide (NT-proBNP) concentrations assessed in the 11 outlier dogs at first sampling and 3 months later.

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 $\pm$ SD 5.8 $\pm$ 2.9	Mean $\pm$ SD 30.0 $\pm$ 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)
Kelliham 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, crossbreeds, 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 $\pm$ SD 9.1 $\pm$ 1.6	Mean $\pm$ SD 6.8 $\pm$ 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]
Kelliham 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; Kelliham 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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