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# Breed Differences in Natriuretic Peptides in Healthy Dogs

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Breed Differences in Natriuretic Peptides in Healthy Dogs  
Rasskillnader i natriuretiska peptider hos friska hundar

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## ABBREVIATIONS

ANP	Atrial Natriuretic Peptide
BCS	Body Condition Score
BNP	B-type Natriuretic Peptide
CHF	Congestive Heart Failure
IQR	Inter Quartil Range
MMVD	Myxomatous Mitral Valve Disease
NT-proANP	N-terminal fragment of pro-atrial Natriuretic Peptide
NT-proBNP	N-terminal fragment of pro-B-type Natriuretic Peptide
proANP 31-67	Pro-atrial Natriuretic Peptide 31-67

## SUMMARY

Measurement of plasma concentrations of natriuretic peptides have been suggested to be of diagnostic and prognostic value in canine cardiac disease. In several previous canine studies, however, a considerable overlap between investigated groups has been identified. A reliable natriuretic peptide test for use in clinical practice requires establishment of reference values for natriuretic peptides in healthy dogs and cut-off values for subclinical disease as well as for clinical signs of congestive heart failure. Numerous physiological, pathological and pharmacological factors, aside heart function, may influence concentration of natriuretic peptides. A potential effect of dog breed on natriuretic peptide concentration has been suggested but has, to our knowledge, not previously been specifically studied. Therefore, the aim of the present study was to investigate breed variation in plasma concentrations of natriuretic peptides in healthy dogs.

Dogs of nine different breeds were examined at five different centers within the EU. A thorough clinical work-up was performed to exclude cardiovascular disease or other organ-related or systemic disease. 535 healthy, privately-owned dogs were included. Blood samples were taken from all dogs, and plasma concentrations of proANP 31-67 and NT-proBNP were analyzed using commercially available ELISA assays. Samples were analyzed at two separate accredited laboratories, one for each peptide.

The results showed an overall significant breed difference for proANP 31-67 ( $P < 0.0001$ ) as well as for NT-proBNP ( $P < 0.0001$ ). Pair-wise comparison between breeds showed significant differences in approximately 50% of the comparisons for both natriuretic peptides. Due to the uneven breed distribution between centers, group-wise comparisons between breeds were performed within each center, again resulting in significant differences in approximately 50% of the comparisons for both natriuretic peptides. The NT-proBNP concentration was associated with gender ( $R^2 = 0.17$ ,  $P < 0.0001$ ) with higher concentrations in female than male dogs. This result should, however, be interpreted cautiously due to uneven gender distribution between centers and high covariance between center, breed and gender.

In conclusion, the study showed considerable breed variation in plasma concentration of natriuretic peptides in healthy dogs. Further studies are needed to establish breed-specific reference values.

## SAMMANFATTNING

Mätning av plasmakoncentration av natriuretiska peptider har föreslagits vara av diagnostiskt och prognostiskt värde för utvärdering av hjärtsjukdom hos hund. I flera tidigare studier har man dock sett en överlappning i uppmätta koncentrationer mellan olika undersökta grupper. För att ett test av natriuretiska peptider ska vara pålitligt för kliniskt bruk krävs fastställda referensvärden för natriuretiska peptider hos friska hundar samt gränsvärden för diagnosticering av hundar med subklinisk hjärtsjukdom respektive hjärtsvikt. Det finns dock ett stort antal fysiologiska, patologiska och farmakologiska faktorer, förutom hjärtsjukdom, som kan påverka plasmakoncentrationen av natriuretiska peptider. Det har föreslagits att hundras kan vara en sådan faktor men detta har till vår kännedom inte tidigare specifikt studerats. Syftet med studien var därför att undersöka rasvariation i plasmakoncentration av natriuretiska peptider hos friska hundar.

Hundar av nio olika raser undersöktes vid fem olika center inom EU. En noggrann hälsoundersökning gjordes för att utesluta hjärtsjukdom eller annan organ-relaterad eller systemisk sjukdom. 535 friska, privatägda hundar inkluderades. Blodprover togs från alla hundar och plasmakoncentration av proANP 31-67 och NT-proBNP analyserades med kommersiella ELISA tester på två auktoriserade laboratorier, ett laboratorium för varje peptid.

Resultaten visade signifikanta skillnader mellan raserna för proANP 31-67 ( $P < 0.0001$ ) och för NT-proBNP ( $P < 0.0001$ ). Parvisa rasjämförelser visade signifikanta skillnader i ungefär 50% av jämförelserna för båda natriuretiska peptider. På grund av den ojämna rasfördelningen mellan centra, gjordes rasjämförelser även inom varje center. Återigen sågs signifikanta skillnader i ungefär 50 % av jämförelserna för de båda natriuretiska peptiderna. Koncentrationen av NT-proBNP var associerad med kön ( $R^2 = 0.17$ ,  $P < 0.0001$ ) med högre koncentrationer hos tikar än hanhundar, men detta resultat bör tolkas med försiktighet pga den ojämna könsfördelningen mellan centra samt den höga kovariansen mellan center, ras och kön.

Sammanfattningsvis visade denna studie påtagliga skillnader i plasmakoncentration av natriuretiska peptider mellan raser. Ytterligare studier behövs för att fastställa rasspecifika referensvärden.



## INTRODUCTION

### Background

Heart disease in dogs is, in general practice, assessed by history of clinical signs, physical examination, auscultation of heart and lungs and radiographic imaging, despite the fact that it is difficult to make detailed assessments of the heart using these techniques (Oyama et al., 2008, Prošek et al., 2007). More advanced diagnostic tools, such as echocardiography, are less accessible, because these methods require a trained clinician as well as expensive equipment (Oyama et al., 2008, Prošek et al., 2007). There are several reasons to strive for a simple, inexpensive and yet accurate blood test to help detect heart disease in dogs (Oyama et al., 2008, Prošek et al., 2007). In an acute clinical situation, it is important to quickly distinguish between clinical signs caused by heart disease, and similar clinical signs from afflictions unrelated to the heart (Oyama et al., 2007, Prošek et al., 2007). It would also be of great value to be able to monitor the progression of heart disease with the aim to accurately assess disease severity and optimize therapeutic intervention. In addition, because some forms of heart diseases can be hereditary, it would be beneficiary to be able to diagnose occult heart disease early to exclude affected dogs from breeding (Oyama et al., 2007).

### Introduction to natriuretic peptides

DeBold *et al* discovered the first cardiac endocrine factor in 1981 when they found atrial natriuretic peptide (ANP) through experiments performed on rats. Their further investigations confirmed that ANP was produced by the heart and had diuretic, natriuretic and hypotensive abilities as well as an inhibitory effect on the renin-angiotensin-aldosterone system (Boomsma and van den Meiracker, 2001, de Bold, 1985, Maisel et al., 2008, Sudoh et al., 1988). Brain natriuretic peptide (BNP) was originally isolated from porcine brain in 1988 by Sudoh *et al*. Structurally akin to ANP, with a sequence homology of 70%, BNP exhibits physiological effects that are very similar to the effects of ANP (Sudoh et al., 1988). It has later been established that BNP is primarily a cardiac peptide which is produced and secreted in a similar fashion to ANP, and it is now also known as B-type natriuretic peptide (Mukoyama et al., 1991, Sagnella, 2001, Saito et al., 1989, Yasue et al., 1994). There is a third member of the family, the C-type natriuretic peptide (CNP). It is structurally similar to ANP but is mainly produced in endothelium and has physiological actions that are distinct from ANP and BNP and will because of this not feature in this thesis (Boomsma and van den Meiracker, 2001, Koller and Goeddel, 1992, Sagnella, 2001, Wei et al., 1993).

ANP and BNP both consist of one central disulphide ring of 17 amino acids with N-terminal and C-terminal segments that have variable lengths (Koller and Goeddel, 1992, Sagnella, 2001). They are synthesized as high molecular weight precursors, pro-hormones, which are cleaved into two segments (Saito et al., 1989, Yasue et al., 1994). One low molecular weight, biologically active segment in the C-terminal region; described as ANP or BNP, and one biologically inactive N-terminal segment; described as NT-proANP or NT-proBNP (Maisel et al., 2008, Saito et al., 1989, Thibault et al., 1987, Yasue et al., 1994). NT-proANP is then further cleaved into three segments, proANP 1-30, 31-67 and 68-98 (Winters et al., 1989). ANP and BNP both bind to the A-type natriuretic peptide receptor which activates guanylyl

cyclase, leading to the formation of the intercellularly active cyclic guanosine monophosphate (cGMP) and thereby evoking cellular response (Boomsma and van den Meiracker, 2001, Koller and Goeddel, 1992). There are two ways of clearance. All of the segments are cleared by enzymatic degradation by neutral endopeptidase and for the biologically active ANP and BNP, there is also a specific clearance receptor (Boomsma and van den Meiracker, 2001). Inactive segments have a longer half-life and longer lasting plasma concentrations because they do not have a specific clearance receptor (Boomsma and van den Meiracker, 2001, Buckley et al., 1999, Morgenthaler et al., 2004).

The main cause of production and release of natriuretic peptides is an increase in stretch and/or pressure in the myocardial wall, although it is worth noting that angiotensin and endothelin are also thought to be influential (Boomsma and van den Meiracker, 2001, Luchner et al., 2000, Yasue et al., 1994). ANP is stored in secretion granules in the myocardial wall, mainly in high molecular mass precursor form, and there is no increase in gene expression in response to myocardial stretch (Mantymaa et al., 1993, Sagnella, 2001). BNP is constitutively secreted and has little intracellular storage, and its gene expression is increased in response to myocardial stretch (Mantymaa et al., 1993, Sagnella, 2001). In healthy humans, ANP is secreted mainly from the atrial tissue and BNP mainly from the left ventricle (Mukoyama et al., 1991). Canine ANP is produced mainly in the atrium as in humans, but in canines the main BNP production is also in the atrial myocardium in the healthy heart (Luchner et al., 1998, Yasue et al., 1994). In CHF, however, ventricular production of ANP and BNP is activated in response to stretching of ventricular myocardial walls (Luchner et al., 1998, Luchner et al., 2000). Davis *et al* (1994) was the first to report that an increase in plasma concentration of BNP could be used as an indicator of heart failure in humans. Further study on humans led to the discovery that the secretion of ANP and BNP from the myocardial wall rises proportionally to cardiac dysfunction (Yasue et al., 1994). Häggström *et al* (1994) was first to report a rising concentration of plasma ANP in dogs with increasing heart disease severity, and Asano *et al* (1999) concluded early that ANP and BNP could be useful in diagnosing CHF in dogs. Plasma concentrations of N-Terminal segments have also been shown to correlate with heart size and systolic function in dogs (Oyama et al., 2007, Oyama et al., 2008).

## Tests

Early tests for measuring natriuretic peptides in humans and dogs concentrated on ANP and BNP analysis (Boswood et al., 2008, Sagnella, 2001). In the beginning specific radio immune assays (RIAs) were used and by the late 1990s, immunoradioassay (IRMA) was developed (Boswood et al., 2008, Sagnella, 2001). The aminoacid sequences in human and canine natriuretic peptides are different which makes it difficult to use human assay methods for measuring canine natriuretic peptides (Boswood et al., 2008). This difference is greater for BNP and NT-proBNP than for ANP and NT-proANP to the extent that a human test may be used for canine NT-proANP, but a specific canine test needs to be used for canine NT-proBNP (Boswood et al., 2008, O'Sullivan et al., 2007). Canine specific competitive enzyme-linked immunosorbent assays (ELISA) for ANP and BNP, respectively, are now in use (DeFrancesco et al., 2007).

It is widely accepted that increased blood concentrations of ANP, BNP, NT-proANP and NT-proBNP are all indicative of heart disease in both dogs and humans (Oyama et al., 2007, Oyama et al., 2008, Sagnella, 2001). There has been a lot of discussion as to which peptide (ANP or BNP) and which segment (biologically active or inactive form) is the best for evaluating or diagnosing heart disease (Sagnella, 1998). Different forms might be better for different heart afflictions, and since the inactive segments have been found to have longer half-life than the biologically active segments; they may be better suited as diagnostic tools (Buckley et al., 1999, Morgenthaler et al., 2004, Pemberton et al., 2000, Sagnella, 1998).

When compared to ANP, BNP has been shown to have greater sensitivity and specificity as an indicator of heart failure in humans as well as in dogs (Cowie et al., 1997, Oyama et al., 2007). BNP also has better characteristics for a screening test (Cowie et al., 1997, O'Sullivan et al., 2007). It has both greater specificity and sensitivity than ANP, for several different cut-off values, and has also shown a higher predictive value (Chetboul et al., 2004, Cowie et al., 1997). As for practicality as a clinical test, BNP has better stability in whole blood in room temperature than ANP (Cowie et al., 1997).

BNP and NT-proBNP have different half-lives, modes of degradation and cut-off values (Maisel et al., 2008, Sagnella, 1998). The half-life for BNP in dogs is 1.5 minutes, compared to 22 minutes in humans (Thomas and Woods, 2003). To our knowledge, the half-life of NT-proBNPs in dogs has not been determined, but in sheep it has been shown to be 15 times longer than that of BNP (Pemberton et al., 2000). The inactive segments of proANP have also been shown to have longer half-lives than ANP in humans (Buckley et al., 1999, Morgenthaler et al., 2004). A short half-life in a peptide might indicate that significant degradation can occur before it can be measured; hence the inactive fragments might be better as diagnostic tools in dogs. BNP might however be better suited for detecting short-term changes than NT-proBNP (Oyama et al., 2008). When compared to NT-proBNP tests, BNP tests need specialized collection and handling which makes NT-proBNP testing easier to execute (Oyama et al., 2008).

### **Natriuretic peptides today**

In the year 2000, the Food and Drug Administration (FDA) approved BNP as an aid in the diagnosis of human heart failure. BNP and NT-proBNP tests are currently widely used in human medicine for diagnosis, risk stratification and therapeutic decision making in acute coronary syndromes, hypertension, pulmonary embolism and early detection of ventricular and atrial myocardial dysfunction that might lead to heart failure (Boomsma and van den Meiracker, 2001, Maisel et al., 2004, Maisel et al., 2008). In the diagnosis of acute CHF, a quick result is crucial and so called bedside tests are used (Maisel et al., 2004, Maisel et al., 2008). There are no bedside tests available for dogs as of yet, and natriuretic peptide assay is not yet standard in veterinary practice (Ettinger et al., 2012). However, elevated plasma concentrations of natriuretic peptides have been identified in dogs with dilated cardiomyopathy (DCM) (Haggstrom et al., 2000, O'Sullivan et al., 2007, Oyama et al., 2007, Oyama et al., 2008, Singletary et al., 2012, Wess et al., 2011). In dogs with myxomatous mitral valve disease (MMVD), concentrations of natriuretic peptides have been shown to

increase, partly depending on disease severity (Oyama et al., 2008, Takemura et al., 2009, Tarnow et al., 2009, Wolf et al., 2012), and NT-proBNP has been shown predicative of outcome in dogs with MMVD (Reynolds et al., 2012, Tarnow et al., 2009). Natriuretic peptides have also been found useful to distinguish CHF from respiratory disease (Boswood et al., 2008, DeFrancesco et al., 2007, Prošek et al., 2007, Reynolds et al., 2012). To have a relevant diagnostic and prognostic test for heart disease, it is necessary to establish an upper reference limit for natriuretic peptides in healthy individuals, and cut-off values for clinical signs of heart disease (Ettinger et al., 2012, Sagnella, 1998). A considerable overlap between concentrations in investigated groups has however been identified between and within several of the canine studies.

### **Influencing factors**

There are numerous physiological, pathological, pharmacological, biochemical and hematological factors besides heart function that may influence the levels of natriuretic peptides in humans as well as in dogs (Balion et al., 2008, Boswood et al., 2008, DeFrancesco et al., 2007, Ettinger et al., 2012, Kellihan et al., 2009, O'Sullivan et al., 2007, Oyama et al., 2008, Prošek et al., 2007, Takemura et al., 2009, Tarnow et al., 2009). In a review article, Balion *et al* (2008) list no fewer than 103 factors that affect human BNP or NT-proBNP. Another important consideration is that natriuretic peptides are involved in several different physiological processes in the body and their levels may be affected by several different factors (Balion et al., 2008). There are concerns of how to adjust reference limits and cut-off-values in regard to the large number of affecting factors in humans as well as in dogs (Balion et al., 2008, Boswood et al., 2008, DeFrancesco et al., 2007, Ettinger et al., 2012, Kellihan et al., 2009, Oyama et al., 2008, Prošek et al., 2007, Takemura et al., 2009, Tarnow et al., 2009, O'Sullivan et al., 2007). In addition, the ideal sensitivity and specificity for a test is still debated (Ettinger et al., 2012, Kellihan et al., 2009, Maisel et al., 2008, Singletary et al., 2012).

Age is an important influential physiological factor in humans, potentially caused by decreased heart function with greater age (Balion et al., 2008). According to Balions *et al* (2008) review article, age was positively associated with both BNP and NT-pro-BNP in 80 % of the reviewed studies. However, the majority of studies performed on dogs did not find an association between natriuretic peptide plasma concentration and age (Boswood et al., 2008, DeFrancesco et al., 2007, Eriksson et al., 2001, Kellihan et al., 2009, Oyama et al., 2007, Oyama et al., 2008, Tarnow et al., 2009). In the few canine studies that did find an association, it was suggested that the association could be caused by the higher incidence of heart disease in older male dogs (DeFrancesco et al., 2007, Eriksson et al., 2001, Ettinger et al., 2012, O'Sullivan et al., 2007). Women had a higher concentration of plasma BNP than men in around 50% of human studies (Balion et al., 2008). Most canine studies that included gender and reproductive status did not find an association (Boswood et al., 2008, DeFrancesco et al., 2007, Eriksson et al., 2001, Ettinger et al., 2012, Oyama et al., 2008, Tarnow et al., 2009), but one study found a positive association between ANP and age (O'Sullivan, et al., 2007). In humans, body weight has been shown to correlate inversely with plasma concentration of natriuretic peptides possibly caused in part by progressive weight

loss in connection with heart disease (Chen-Tournoux et al., 2010, Eriksson et al., 2001). Canine studies show conflicting results (Eriksson et al., 2001, Ettinger et al., 2012, Kellihan et al., 2009, Tarnow et al., 2009). The individual weekly variability of plasma NT-pro-BNP concentration has been found to be considerable for humans as well as for dogs (Kellihan et al., 2009, O'Hanlon et al., 2007, Oyama et al., 2008).

To our knowledge, variation in natriuretic peptide concentration between dog breeds has not previously been specifically studied. However, significantly higher serum concentration of NT-proBNP has been found in healthy pure-breed dogs compared to concentrations in healthy mixed-breed dogs (Oyama et al., 2008). The possibility of biological differences in production, release and clearance of NT-proBNP between breeds has been suggested (Oyama et al., 2008). Hence, the aim of the present study was to investigate breed variation in plasma concentrations of natriuretic peptides in healthy dogs.

## **MATERIALS AND METHODS**

### **Animals**

To be included in the study, dogs had to be pure-bred, healthy, and between 1 and 7 years of age. These dogs had been found healthy, which in this study entailed passing a general health examination with no abnormal findings, no ECG abnormalities were detected, and all echocardiographic variables were within reference values. Furthermore, no clinically significant abnormalities were detected in the hematological or blood biochemistry variables. They also had to have a normal body condition score, and could not be related to each other at parental level. Dogs were examined at five different centers, as part of the EU funded LUPA-project (Lequarre et al., 2011). Centers included University of Liège, Belgium; University of Copenhagen, Denmark; National Veterinary School of Alfort, France; University of Helsinki, Finland and the Swedish University of Agricultural Sciences, Sweden. The study was approved by an ethical committee in each participating country. Dogs were privately-owned and informed owner consent was obtained. Each cohort included dogs of one gender only, either intact males or females which were spayed or in anestrus. Exclusion criteria consisted of any finding indicating systemic or organ related disease observed at the clinical examination outlined below.

### **Preparations**

Dog owners were instructed to solely feed their dog standard commercial dog food two weeks prior to participation in the study to avoid uneven or excessive salt intake. On examination day, all dogs were fasting and had no access to water for at least two hours before the examination.

## Verification of health status

Each dog underwent a general physical examination including blood pressure measurement by high-definition oscillometry, a five minute ECG registration and an echocardiographic examination. The echocardiographic examination was performed from the right and left side using standardized imaging planes (Thomas and Woods, 2003) and continuous ECG-monitoring. The left atrial to aortic root ratio was quantified from the right two-dimensional short-axis view (Hansson et al., 2002). Pulmonic and aortic flow velocities were measured by spectral Doppler, and the mitral, aortic, pulmonic and tricuspid valves were screened using color Doppler. The left ventricle was measured using standard M-mode techniques. Urine samples were collected by natural micturition and standard urine analysis was performed by dipstick and refractometer. Blood sampling was carried out by venipuncture and blood was collected into 5-mL EDTA- and serum tubes. Analysis of hematology and biochemistry regarding liver and kidney variables, glucose and electrolytes were performed.

## Analysis of natriuretic peptides

Concentrations of proANP 31-67 and NT-proBNP were measured in plasma. EDTA-tubes were centrifuged within 30 minutes of blood sample collection. Plasma was harvested, transferred into plastic cryotubes and samples were frozen. Samples were stored at -80°C. At one center, samples were, for practical reasons, stored at -20°C for a maximum of 2 weeks, where after they were transferred frozen to -80°C and stored for batched analysis. Previous studies have shown that midregional proANP is stable for 3-6 months at -20°C and NT-proBNP is stable for 4 months at -20°C (Hunt et al., 1997, Morgenthaler et al., 2004, Mueller et al., 2004). All samples were later transported frozen to two accredited laboratories, which analyzed concentrations of proANP 31-67 and NT-proBNP, respectively, using commercially available ELISA assays<sup>1,2</sup>. All samples were analyzed in duplicate and the mean of the two samples were used for data analysis.

## Statistical analysis

Commercially available software<sup>3</sup> was used for all statistical analyses. Data is presented as medians and interquartile ranges (IQR). A value of  $P < 0.05$  was considered significant for the analysis, unless otherwise indicated.

The non-parametric Kruskal-Wallis test was used to investigate overall differences between breeds, in concentrations of proANP 31-67 and NT-proBNP, respectively. If a significant difference was detected, pair-wise comparisons between breeds were performed by use of the Mann Whitney U-test with Bonferroni adjustment, for which a value of  $P < 0.0014$  was considered significant. The Kruskal-Wallis test was also used to investigate differences between breeds within each center, for concentrations of proANP 31-67 and NT-proBNP, respectively. In centers including more than two breeds, pair-wise comparisons between

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<sup>1</sup> NT-proBNP: ELISA Cardiopet proBNP test (IDEXX Laboratories, Westbrook, Maine, USA)

<sup>2</sup> ProANP 31-67: ELISA VETSIGN Canine CardioSCREEN (Biomedica, Vienna, Austria)

<sup>3</sup> JMP, version 9.0.0, SAS Institute Inc, Cary, NC, USA

breeds were performed by use of the Mann Whitney U-test with Bonferroni adjustment, if an overall significant difference was detected.

Unilinear regression analyses were performed to evaluate potential associations between breed as well as age, gender, body weight, examination center, and concentrations of proANP 31-67 and NT-proBNP, respectively. A subanalysis of the same variables by unilinear regression analysis was performed in the Labrador retriever breed alone, since it included the largest number of dogs, was represented at four out of five centers and included both female and male dogs.

To compensate for other confounding factors for the breed variable on plasma concentration of natriuretic peptides, a multiple regression analysis was performed, including variables which reached  $P < 0.2$  in the unilinear regression analysis. Analyses were performed in a reverse stepwise manner (Bland, 1995), starting with all variables included in the model and then removing the variable with the highest  $P$ -value until all remaining variables had a value of  $P < 0.05$ . All variables were assessed only as main effects; no interaction terms were considered in the model.

The distribution of residuals in the multiple-regression analysis was tested for normality using Shapiro Wilk  $W$  test. The adjusted  $R^2$  is defined as the percentage of the total sum of squares that can be explained by the regression and it also considers the degrees of freedom for variables added. No multiple regression analysis was performed in the Labrador retriever cohort, due to the high covariance between center and gender.

## RESULTS

### Distribution

In total, 535 dogs of nine breeds were included in the study. Distribution of breeds and individuals examined at the different centers are shown in table 1. Each center examined dogs belonging to 2-4 different breeds, some breeds were shared between centers. Gender distribution was uneven with 416 male and 119 female dogs (Table 1). All males were intact while females were spayed or in anoestrus. Median age (n = 531) was 3.3 (IQR 2.6-4.4) years and median body weight (n = 497) was 30.2 (IQR 23.5-36.0) kg.

Table 1. Distribution by center of examination, breed, and gender

	Belgium	Denmark	Finland	France	Sweden	Total
<b>Box</b>					15M	<b>15</b>
<b>BS</b>	97M			26M		<b>123</b>
<b>CKCS</b>					34M	<b>34</b>
<b>Dach</b>			26M		16M	<b>42</b>
<b>Dob</b>				25M		<b>25</b>
<b>FinL</b>			50M			<b>50</b>
<b>GS</b>	16M		60M			<b>76</b>
<b>Lab</b>	6M	45F		29F	45M	<b>125</b>
<b>NF</b>		45F				<b>45</b>
<b>Total</b>	<b>119</b>	<b>90</b>	<b>136</b>	<b>80</b>	<b>110</b>	<b>535</b>

Box = Boxer, BS = Belgian Shepherd, CKCS = Cavalier King Charles Spaniel, Dach = Dachshund, Dob = Doberman, FinL = Finnish Lapphund, GS = German Shepherd, Lab = Labrador Retriever, NF = Newfoundland. M = male, F = female

### Natriuretic peptides

The median concentration of proANP 31-67 (n = 535) was 889 (IQR 735-1065) pmol/L, while the median concentration of NT-proBNP (n = 527) was 638 (IQR 403-980) pmol/L. Natriuretic peptide concentrations by breed are shown in Figure 1 and Figure 2.

### Group wise comparisons, all dogs

There was an overall significant breed difference for proANP 31-67 ( $P < 0.0001$ ) and NT-proBNP ( $P < 0.0001$ ). Pair-wise comparisons between breeds showed significant differences for 15 of 36 comparisons of proANP 31-67 and 18 of 36 comparisons of NT-proBNP (Table 3). Concentrations of proANP 31-67 were lowest in Doberman Pinschers, with a median value almost half of the values in German Shepherds and Cavalier King Charles Spaniels, which had the highest concentrations (Figure 1). Concentrations of NT-proBNP were lowest in Dachshunds, whereas Labrador Retrievers and Newfoundlands had the highest concentrations with median values of three times the median concentration in Dachshunds (Figure 2).



## ProANP 31-67 by breed

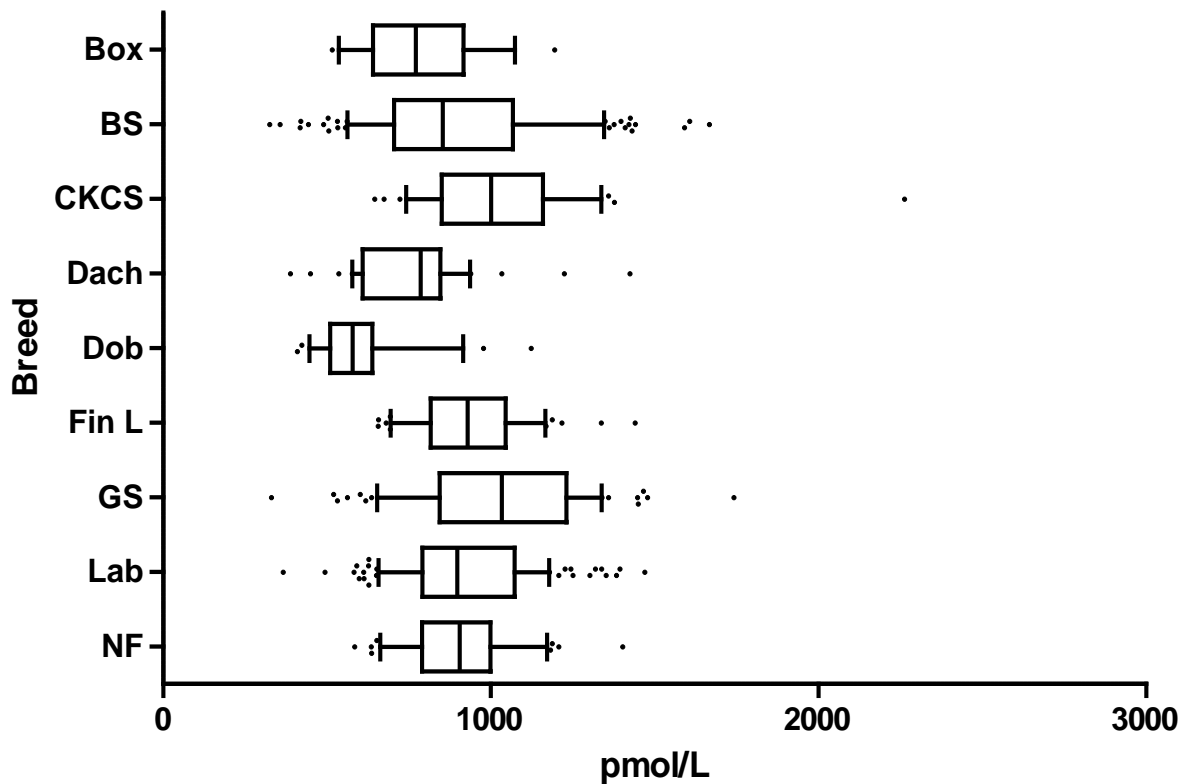


Figure 1. Boxplot showing distribution of proANP 31-67 by breed. The top, bottom and line through the middle of the box correspond to the 75<sup>th</sup> percentile (top quartile), the 25<sup>th</sup> percentile (bottom quartile) and the 50<sup>th</sup> percentile (median), respectively. The whiskers extend from the bottom 10<sup>th</sup> percentile (bottom decile) to the top 90<sup>th</sup> percentile (top decile). Outliers, represented by black dots, show the highest and lowest individual concentrations. Outliers were not excluded from the statistical analyses. There was an overall significant difference between breeds ( $P < 0.0001$ ). For information of which breeds that differed significantly, see table 5. Box = Boxer, BS = Belgian Shepherd, CKCS = Cavalier King Charles Spaniel, Dach = Dachshund, Dob = Doberman, FinL = Finnish Lapphund, GS = German Shepherd, Lab = Labrador Retriever, NF = Newfoundland

## NT-proBNP by breed

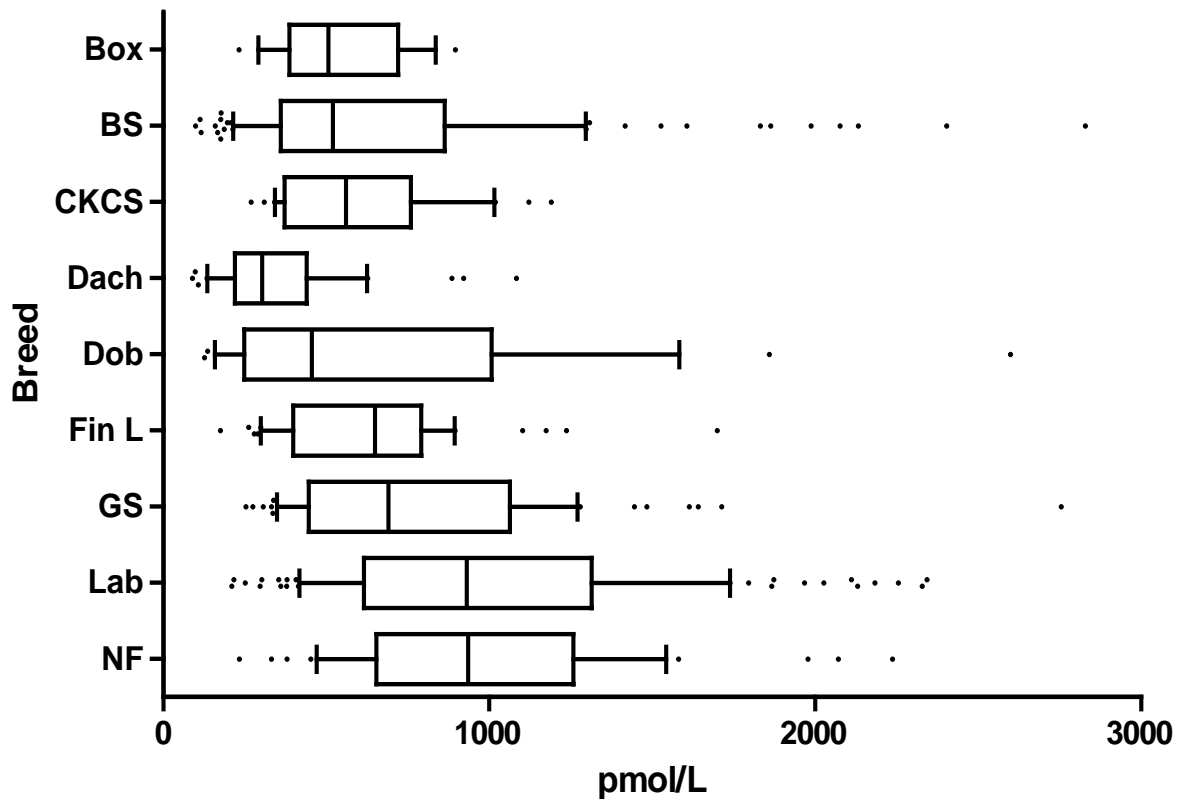


Figure 2. Boxplot showing distribution of NT-proBNP by breed. The top, bottom and line through the middle of the box correspond to the 75<sup>th</sup> percentile (top quartile), the 25<sup>th</sup> percentile (bottom quartile) and the 50<sup>th</sup> percentile (median), respectively. The whiskers extend from the bottom 10<sup>th</sup> percentile (bottom decile) to the top 90<sup>th</sup> percentile (top decile). Outliers, represented by black dots, show the highest and lowest individual concentrations. Outliers were not excluded from the statistical analyses. There was an overall significant difference between breeds ( $P$ -value 0.0001). For information of which breeds that differed significantly, see table 5. Box = Boxer, BS = Belgian Shepherd, CKCS = Cavalier King Charles Spaniel, Dach = Dachshund, Dob = Doberman, FinL = Finnish Lapphund, GS = German Shepherd, Lab = Labrador Retriever, NF= Newfoundland

## Plasma concentrations of natriuretic peptides by breed

Table 2. Plasma concentrations of natriuretic peptides by breed, median values and inter quartil range (IQR) (pmol/L)

Breed	proANP 31-67			NT-proBNP		
	n=	Median	IQR	n=	Median	IQR
<b>Box</b>	15	771	640-917	15	506	387-720
<b>BS</b>	123	853	705-1067	122	521	360-863
<b>CKCS</b>	34	1001	850-1159	34	561	373-1016
<b>Dach</b>	42	786	609-846	42	303	220-440
<b>Dob</b>	25	571	497-638	25	452	224-937
<b>FinL</b>	50	930	816-1045	50	650	399-791
<b>GS</b>	76	1034	844-1231	75	691	446-1063
<b>Lab</b>	125	898	791-1073	119	931	616-1314
<b>NF</b>	45	905	790-999	45	935	654-1259

Box = Boxer, BS = Belgian Shepherd, CKCS = Cavalier King Charles Spaniel, Dach = Dachshund, Dob = Doberman, FinL = Finnish Lapphund, GS = German Shepherd, Lab = Labrador Retriever, NF= Newfoundland.

## Pair wise breed comparison

Table 3. Pair-wise comparisons between breeds in plasma concentrations of natriuretic peptides showing the significant P-values for each peptide

	Box	CKCS	Dach	Dob	FinL	GS	Lab	NF	
<b>BS</b>				<.0001		0.0008		proANP 31-67	
			<.0001				<.0001	<.0001	NT-proBNP
<b>Box</b>		0.0014				0.0004		proANP 31-67	
			0.0013				<.0001	0.0002	NT-proBNP
<b>CKCS</b>			<.0001	<.0001				proANP 31-67	
			<.0001				<.0001	<.0001	NT-proBNP
<b>Dach</b>				0.0002	<.0001	<.0001	<.0001	0.0002	proANP 31-67
					<.0001	<.0001	<.0001	<.0001	NT-proBNP
<b>Dob</b>					<.0001	<.0001	<.0001	<.0001	proANP 31-67
							0.0002	0.0009	NT-proBNP
<b>FinL</b>								proANP 31-67	
							<.0001	<.0001	NT-proBNP
<b>GS</b>								proANP 31-67	
							0.0006		NT-proBNP
<b>Lab</b>								proANP 31-67	
									NT-proBNP

Box = Boxer, BS = Belgian Shepherd, CKCS = Cavalier King Charles Spaniel, Dach = Dachshund, Dob = Doberman, FinL = Finnish Lapphund, GS = German Shepherd, Lab = Labrador Retriever, NF= Newfoundland

### Group wise comparisons, within center

Overall significant breed differences were found within three of the five centers for proANP 31-67 ( $P \leq 0.0006$ ) and four of five centers for NT-proBNP ( $P < 0.003$ ). Pair-wise comparisons showed significant differences for 8 of 16 comparisons of proANP 31-67 and 7 of 16 comparisons of NT-proBNP.

### Unilinear regression analysis

In the unilinear regression analysis of all dogs (Table 4), an association was shown between proANP 31-67 and breed, as well as center of examination. NT-proBNP was also associated with breed and center of examination. Furthermore, NT-proBNP was associated with increasing body weight and with gender, where higher concentrations were found in female than in male dogs. In the separate unilinear regression analysis of Labrador retrievers (Table 5), an association was shown between proANP 31-67 and center of examination, as well as gender, with higher concentrations in male than female dogs. NT-proBNP was associated with center of examination and gender, with higher concentrations in female than male dogs (Table 5), (Figure 3).

Table 4. Significant outcomes in the unilinear regression analysis of all dogs. An empty space indicates that no significant association was found

	proANP 31-67		NT-proBNP	
	R <sup>2</sup>	P-value	R <sup>2</sup>	P-Value
<b>Body weight</b>			0.04	<0.0001
<b>Breed</b>	0.15	<0.0001	0.16	<0.0001
<b>Center of examination</b>	0.07	<0.0001	0.16	<0.0001
<b>Gender</b>			0.17	<0.0001

Table 5. Significant outcomes in the separate unilinear regression analysis of Labrador Retriever cohorts. An empty space indicates that no significant association was found

	proANP 31-67		NT-proBNP	
	R <sup>2</sup>	P-value	R <sup>2</sup>	P-Value
<b>Age</b>				
<b>Center of examination</b>	0.11	0.003	0.24	<0.0001
<b>Gender</b>	0.05	0.01	0.24	<0.0001

## NT-proBNP in Labrador retrievers by gender

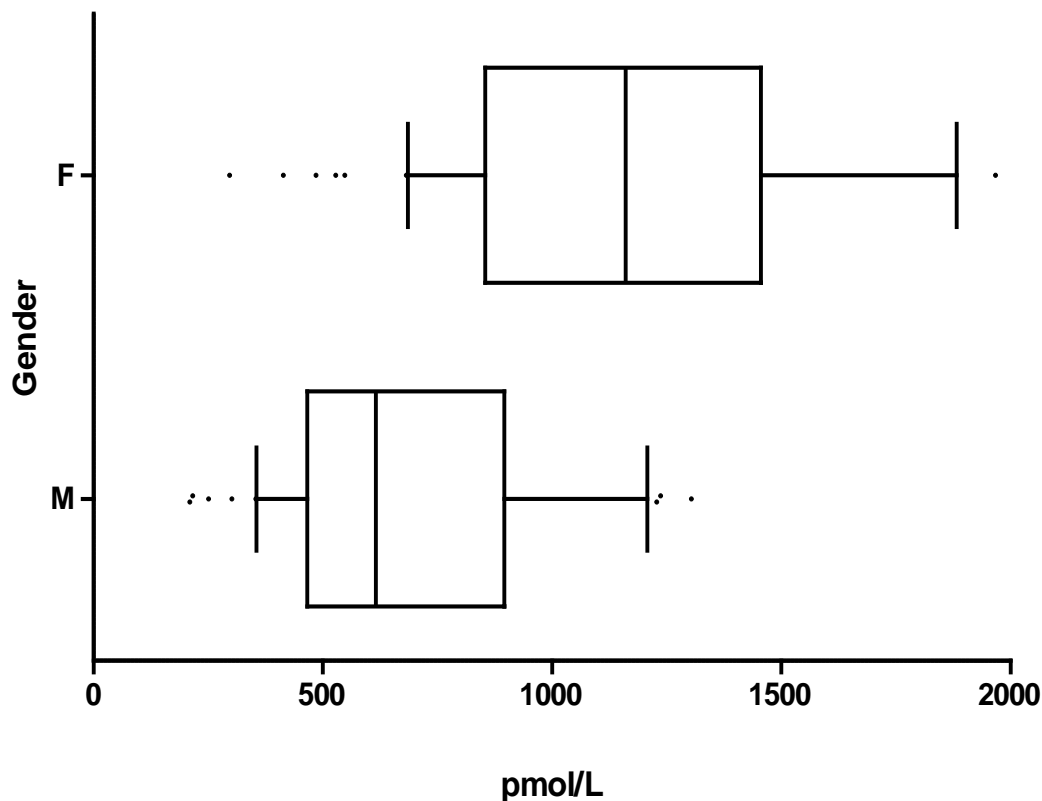


Figure 3. Boxplot showing the NT-proBNP concentration in female ( $n=68$ ) and male ( $n=51$ ) Labrador Retriever dogs. The top, bottom and line through the middle of the box correspond to the 75<sup>th</sup> percentile (top quartile), the 25<sup>th</sup> percentile (bottom quartile) and the 50<sup>th</sup> percentile (median), respectively. The whiskers extend from the bottom 10<sup>th</sup> percentile (bottom decile) to the top 90<sup>th</sup> percentile (top decile). Outliers, represented by black dots, show the highest and lowest individual concentrations. Outliers were not excluded from the statistical analyses. (F = females, M = males)

### Multiple regression analysis

The multiple regression analysis of all dogs confirmed an effect of breed ( $P<0.0001$ ) and center ( $P = 0.0008$ ) on proANP 31-67 concentration with an adjusted model  $R^2$  of 0.16. Also for NT-proBNP concentration, multiple regression analysis confirmed an effect of breed ( $P<0.0001$ ) and center ( $P<0.0001$ ) with an adjusted model  $R^2$  of 0.22.

## DISCUSSION

The main findings of this study were highly significant overall breed differences in plasma concentrations of proANP 31-67 and NT-proBNP in healthy dogs examined at five centers. Significant differences were found in approximately fifty percent of the pair-wise comparisons between breeds for proANP 31-67 as well as for NT-proBNP concentrations, when including all centers and also within each center. Among the nine breeds included in the study, Labrador Retrievers and Newfoundlands had the highest median NT-proBNP concentrations with values three times as high as Dachshunds. Interestingly, in the case of proANP 31-67, it was instead German Shepherds and Cavalier King Charles Spaniels that had the highest median concentrations, which were twice the median concentration in Doberman Pinschers, which had the lowest concentrations. This might be indicative of breed differences in the interrelationship between NT-proBNP and proANP 31-67, potentially due to breed differences in production, release and/or clearance.

A strong selection for certain physiological, morphological and behavioral traits has created dog breeds with a unique diversity among mammalian species, and specific features are inherited closely within a breed (Parker and Ostrander, 2005). The breeds included in the present study represent dogs of varying body sizes, temperament, utilities, and genetic background. Breed differences in, for example, blood pressure, heart rate, and catecholamine concentrations have been found in previous studies, and potential connections to differences in temperament and reactivity pattern in the sympathetic nervous system have been suggested (Bodey and Michell, 1996, Hansson et al., 2002, Hoglund et al., 2012, Rasmussen et al., 2011).

Center of examination was associated with plasma concentration of both proANP 31-67 and NT-proBNP in the unilinear as well as multiple regression analyses. Due to the uneven breed distribution between centers, center was highly covariate with breed, and additional group-wise comparisons were performed between breeds within each center. The results showed a similar percentage of significant pair-wise differences as when all dogs were included (approximately fifty percent for both natriuretic peptides), thus confirming the presence of breed variation in the examined population.

Higher concentrations of NT-pro BNP in female than in male dogs were shown in the statistical analysis of all dogs. An association between gender and concentration of NT-pro BNP was shown in the unilinear regression analysis, but not in the multiple regression analysis. Pro-ANP 31-67 was not associated with gender in the unilinear or multiple regression analysis of all dogs. Since each cohort, according to the study protocol, included only dogs of the same gender, the gender distribution in this study was uneven (*Table 1*). To further investigate the effect of gender, a subanalysis of all Labrador Retrievers was performed, as this was the breed with the most even gender distribution. Furthermore, this breed had the highest number of individuals and was represented at four out of the five centers of examination. In the unilinear regression analysis of the Labrador Retrievers, the association between gender and concentration of NT-pro BNP was confirmed and higher concentrations in female dogs were shown. Slightly higher concentrations of pro-ANP 31-67

were found in Labrador Retriever males compared to females. The association was, however, very weak. Due to the uneven gender distribution between centers of examination, our results on gender differences need to be interpreted cautiously and further studies are needed to assess a potential effect of gender on natriuretic peptide concentration in dogs.

In the present study, no association between age and natriuretic peptide concentration was found which is in accordance with the majority of previous canine studies. However, the present study only included young adult to middle aged dogs, thereby excluding dogs of older age in which increased concentrations of natriuretic peptides have been suggested by a few previous studies (DeFrancesco et al., 2007, Eriksson et al., 2001). Further studies are needed to investigate a potential association between age, and natriuretic peptide concentration in healthy dogs.

No association was found between proANP 31-67 or NT-proBNP and body weight in the multiple regression analysis in the present study. However, there was a weak positive association with NT-proBNP in the unilinear regression analysis. This is not in accordance with the few canine studies that have found an association, in which the associations were negative. However, this has been suggested to be caused by progressive weight loss in connection with heart disease, and because our study population only consisted of healthy dogs with a normal body condition score this does not apply to the present study (Eriksson et al., 2001). In the subanalysis of Labrador retrievers, body weight was not associated with proANP 31-67 or NT-proBNP.

For successful use of natriuretic peptides as diagnostic tools in clinical practice, reference values for healthy individuals as well as cut-off values for cardiac disease need to be established. The dogs included in the present study were confirmed healthy by an extensive health examination and yet the present study shows, in addition to variation between breeds, large variations within breeds. To adjust cut-off points in regard to the numerous influential factors is not straightforward. When compared to reference values suggested by a recent study using the same assay, several individuals in our study were shown to have NT-proBNP concentrations equivalent to, or higher than values proposed indicative of heart disease in dogs (Ettinger et al., 2012). In some breeds, a large share of healthy individuals had NT-proBNP concentrations higher than the suggested reference values, and in other breeds, the major part of individuals were markedly below.

Breed specific guidelines and cut-off points should, in light of the findings of this study, be set according to breed variation (DeFrancesco et al., 2007, Eriksson et al., 2001, Oyama et al., 2007, Oyama et al., 2008). Further research on natriuretic peptides in dogs should preferably be performed on breed matched cohorts. Measurement of plasma natriuretic peptides should not, even when commercially available as a bedside test, be used as a sole diagnostic tool but as an aid in combination with other diagnostic tools.

### **Study limitations**

The study only included nine breeds and therefore the results cannot be considered representative for the entire dog population. Due to the uneven gender representation and narrow age span of included dogs, results should not be seen as reference values. In order to establish such, further studies on present and additional breeds with an even gender and age representation should be performed.

The study included only neutered male dogs and spayed or anoestral females. Therefore, potential differences in natriuretic peptide concentrations between different reproductive statuses could not be assessed. No consideration was given to individual variability in natriuretic peptide concentrations and blood was sampled only once from each dog.

All dogs were fed commercial dog food, but differences in salt intake can have occurred, and this could potentially have affected blood test results.

Sample handling was standardized, although minor differences in short-term freezing temperature occurred at one center. However, natriuretic peptides have been shown stable at the freezing temperatures used (Hunt et al., 1997, Morgenthaler et al., 2004, Mueller et al., 2004), and all samples were analyzed in batches at the same laboratory. Hence, it is unlikely that this should have affected the results.

### **Conclusion**

A considerable breed variation exists in concentrations of proANP 31-67 and NT-proBNP in healthy dogs. Further studies are warranted in order to establish breed-specific reference values.

### **Conflict of interest**

IDEXX laboratories have provided the assays used in the study free of charge. Neither author nor supervisors are connected to IDEXX.



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