

Measuring NT-proBNP in small animal practice

A study of pre-analytical variables and their effect on measured concentration of feline and canine N-terminal-pro-B-type natriuretic peptide

INTRODUCTION

Heart disease is a common cause of morbidity and mortality in cats and dogs. Small animal clinicians are accustomed to diagnosing and managing advanced stages of heart disease with overt signs of congestive heart failure; however, the diagnosis and management of earlier stages of heart disease and occult heart disease is more challenging.

The general veterinary practitioner has the difficult task of identifying, managing and advising on prognosis of heart disease, often with limited access to diagnostic equipment, or with limited experience in performing diagnostic procedures such as echocardiography. Financial resources are often limited. Furthermore pet owners may be reluctant to allow diagnostic investigation of animals they perceive as healthy, or may be frustrated when diagnostic investigation yields equivocal findings.

A simple and inexpensive diagnostic test, with high sensitivity and specificity, which could provide the small animal general practitioner with reliable diagnostic and prognostic information about heart disease, would be a useful tool. Circulating markers of heart disease in blood (cardiac biomarkers) have shown promise for this purpose and have been the subject of considerable interest in the veterinary literature; however, their utility remains largely untested outside research establishments. Despite this limitation cardiac biomarkers are commercially available and already promoted as diagnostic and prognostic tools to general veterinary practitioners.

The purpose of this study was to test the most commonly used cardiac biomarker in field conditions and to determine if, and to what extent, pre-analytical variables might affect quantitative results.

Heart disease in small animal practice

Prevalence, morbidity and mortality of heart disease in cats and dogs

Cats

Cardiomyopathy, principally hypertrophic cardiomyopathy, is the most common form of heart disease in cats (Riesen *et al.*, 2007). A necropsy study of 4933 cats showed a 5.2% incidence of hypertrophic cardiomyopathy (Liu, 1977). However, prevalence of hypertrophic cardiomyopathy (HCM) or left ventricular hypertrophy (LVH) in asymptomatic feline populations may be significantly higher than previously thought. One study found evidence of hypertrophic cardiomyopathy in 15/103 (14.6%) of apparently healthy cats (Paige *et al.*, 2009). Another study found LVH in 18-62% of 92 normal cats, using various commonly employed criteria to define LVH (Wagner *et al.*, 2010). Furthermore, both these studies showed auscultation was a relatively poor screening tool for HCM in cats.

Data for feline death secondary to heart disease has not been reported; however, mortality appears to be significantly lower than morbidity, indicating cardiomyopathy may be a benign disease for many cats.

Nonetheless feline cardiomyopathy is associated with high morbidity in many affected individuals.

Dogs

Myxomatous mitral valve degeneration (MMVD) is the most common form of heart disease in dogs, encountered principally in small breeds (Detweiler and Patterson, 1965; Thrusfield *et al.*, 1985). The prevalence of MMVD is high, with 58% of dogs older than 9 years showing advanced mitral degeneration at necropsy in one study (Whitney, 1974). A number of studies have indicated particularly high prevalence in predisposed breeds, for example 52% to 59% in Cavalier King Charles Spaniels (CKCS) older than 4 years (Darke, 1987; Beardow and Buchanan, 1993; Haggstrom *et al.*, 2002) and 29% of Dachshunds (Olsen *et al.*, 1999).

Dilated cardiomyopathy (DCM), although less common than MMVD (Buchanan 1999), has strong breed predilection (Martin *et al.*, 2009; Wess *et al.*, 2009; Martin *et al.*, 2010) with particularly high prevalence in some breeds, notably the Doberman Pinscher, where Wess *et al.* (2010) reported a cumulative prevalence of 58.2% in 412 dogs.

A recent large survey of mortality in 15,881 purebred dogs in the UK found that 11% of mortalities were attributed to heart disease (Adams *et al.*, 2010). Although definitive diagnosis was not an inclusion criteria, percentage mortality attributed to heart disease was similar to that reported in previous studies where cardiac diagnosis was known (Detweiler and Patterson, 1965; Fioretti 1988; Buchanan 1992; Bonnett *et al.*, 2005). Adams *et al.*, (2010) found mortality attributed to cardiac disease was particularly high in certain breeds, for example the Scottish Deerhound (24%; 95% CI 19.4% - 29.4%), English Bulldog (20%; 95% CI 14.2%-25.8%), Doberman pinscher (15%; 95% CI 8% - 22%) and Newfoundland (16%; 95% CI 11.6% - 20.4%). Percentage mortality was not reported in the CKCS.

Managing heart disease in cats and dogs poses a variety of challenges for small animal practitioners

When physical evidence of common conditions such as MMVD in dogs is first detected, the clinician will be aware that disease may progress slowly, becoming clinically evident many years later, or that the animal may never develop heart failure (Borgarelli *et al.* 2008, Payne *et al.* 2010). For this reason diagnostic investigation is commonly deferred until such time that signs of heart failure, for example cough or breathlessness, develop. Overt congestive heart failure (CHF), if and when it occurs, is often correctly diagnosed and treated without diagnostic investigation in small animal practice; however, erroneous diagnosis of CHF and inappropriate treatment occurs commonly.

Identification of occult heart disease, where physical signs are not apparent on examination, is rarely achieved in general small animal practice. Occult heart disease is of particular concern in the feline population, where catastrophic consequences such as aortic thromboembolism can occur without warning and prophylactic interventions might have been life saving. DCM, with high prevalence and mortality in certain dog breeds, is often a cause of concern to pet owners and breeders. Practitioners asked to screen pre-disposed animals for occult disease may be unable to find evidence, particularly in the earlier stages of disease.

Potential value of a quantitative test of heart disease in small animal practice

For a variety of reasons including expense and owner apprehension, many animals with heart disease never receive adequate diagnostic investigation. Blood tests are more readily acceptable to pet owners because do not require hospital admission and are relatively inexpensive. Blood markers for heart disease, provided they were reliable, inexpensive, and did not require onerous sample preparation and shipping conditions (or could be used at point-of-care) would likely enjoy widespread use in small animal practice.

Utility of cardiac biomarkers for diagnosis and management of heart disease

Heart disease results in a variety of neuroendocrine responses leading to elevated circulating concentration of vasoactive substances. Measuring circulating concentrations of these substances (cardiac biomarkers) can provide useful clinical information. Many biomarkers of cardiac disease have been identified, but it has been natriuretic peptides, particularly brain natriuretic peptide (BNP) and N-terminal pro-B-type natriuretic peptide (NT-proBNP) that have proved the most clinically useful in human and veterinary medicine to date (Sisson, 2004; Boswood, 2009; Haggstrom et al., 2009; Connolly, 2010; Oyama and Singletary, 2010).

Utility of natriuretic peptides as markers of heart disease in humans

In human medicine, natriuretic peptides have proved reliable and versatile markers of heart disease and heart failure. BNP and NT-proBNP have been used to diagnose CHF in the emergency assessment of dyspnoea, out-performing the clinical judgment of emergency department physicians when diagnosing acute CHF (Maisel *et al.*, 2002 & 2004; Januzzi *et al.*, 2005; Brendan et al., 2006; Januzzi *et al.*, 2008). BNP and NT-proBNP was found to correlate strongly with prognosis and was a powerful independent predictor of mortality in dyspnoeic patients with CHF (Maisel *et al.*, 2004; Masson *et al.*, 2006; Christ *et al.*, 2007; Pfister *et al.*, 2007; Valle *et al.*, 2007). When treating CHF in people, lower mortality was found in NT-proBNP-guided therapy group versus conventionally treated group. This was associated with using higher than normal doses of diuretics and ACE inhibitors to maintain NT-proBNP within target levels (Troughton *et al.*, 2000; Mueller *et al.*, 2004; Richards *et al.*, 2004; Jourdain *et al.*, 2007; Valle *et al.*, 2007; Felker *et al.*, 2009). NT-proBNP has proven utility as a screening tool in asymptomatic humans at risk of developing left ventricular dysfunction (Betti et al. 2009; Troughton and Richards, 2009).

Utility of natriuretic peptides as markers of heart disease in cats and dogs

Although less extensively studied in animals than humans, cardiac biomarkers, particularly natriuretic peptides, have been validated as useful ancillary diagnostic tests when differentiating heart disease from other causes of dyspnoea in cats (Connolly et al., 2008; Wess et al., 2008; Connolly et al., 2009; Fox et al., 2009; Hsu et al., 2009; Ettinger, 2010; Singletary et al., 2011) and dogs (Haggstrom et al., 2000; Prosek et al., 2007; DeFrancesco et al., 2007; Boswood et al., 2008; Fine et al., 2008; Oyama et al., 2008; Achen et al., 2009; Oyama et al., 2009; Tarnow et al., 2009) (see Table A & B in Appendices). Natriuretic peptide concentration correlates with the severity of HCM in cats (MacLean et al., 2006; Connolly et al., 2008; Hsu et al., 2009; Smith, 2009; Zimmering et al., 2009; Ettinger, 2010; Wess et al., 2011) and MMVD in dogs (MacDonald et al., 2003; Achen et al., 2009; Chetboul et al., 2009; Serres et

al.,2009; Tarnow et. al.,2009; Moonamart et al.,2010; Oyama and Singletary, 2010) (see Table C & D in Appendices). A role for natriuretic peptides in identifying occult cardiomyopathy in dogs has been proposed (Chetboul et al., 2004; Oyama et al., 2007), but found to be of limited utility as an isolated test (Baumwart & Meurs, 2005; Wess et al., 2011)(see Table E in Appendices). Although the measurement of NT-proBNP to identify occult heart disease in cats appears promising (Ettinger, 2010; Fox et al.,2011), some studies have showed more limited utility (Hsu, 2009; Singh et al., 2010).

The utility of natriuretic peptides in cats and dogs as a prognostic indicator, or for guiding therapy, has not been thoroughly investigated in either species but some studies show promising utility (Chetboul et al., 2009; Serres et al., 2009; Reynolds et al., 2011; Moonamart et al., 2011).

The NT-proBNP assay

Biology of Natriuretic Peptides

Natriuretic peptides are stored in membrane bound granules in the atria and are released in response to abnormal myocardial wall strain. Proteolytic cleavage of the precursor molecule (proBNP) produces biologically active C-terminal BNP, a 32-amino acid peptide, and a biologically inactive 76-amino acid N-terminal fragment (NT-proBNP). The half-life of C-terminal natriuretic peptides is short (1.57 minutes for canine proBNP; Thomas and Woods, 2003). NT-proBNP is removed more slowly from circulation and its half-life is significantly longer (and plasma concentration significantly higher) than C-terminal BNP, making it a more suitable biomarker for clinical use.

The NT-proBNP assay in cats and dogs

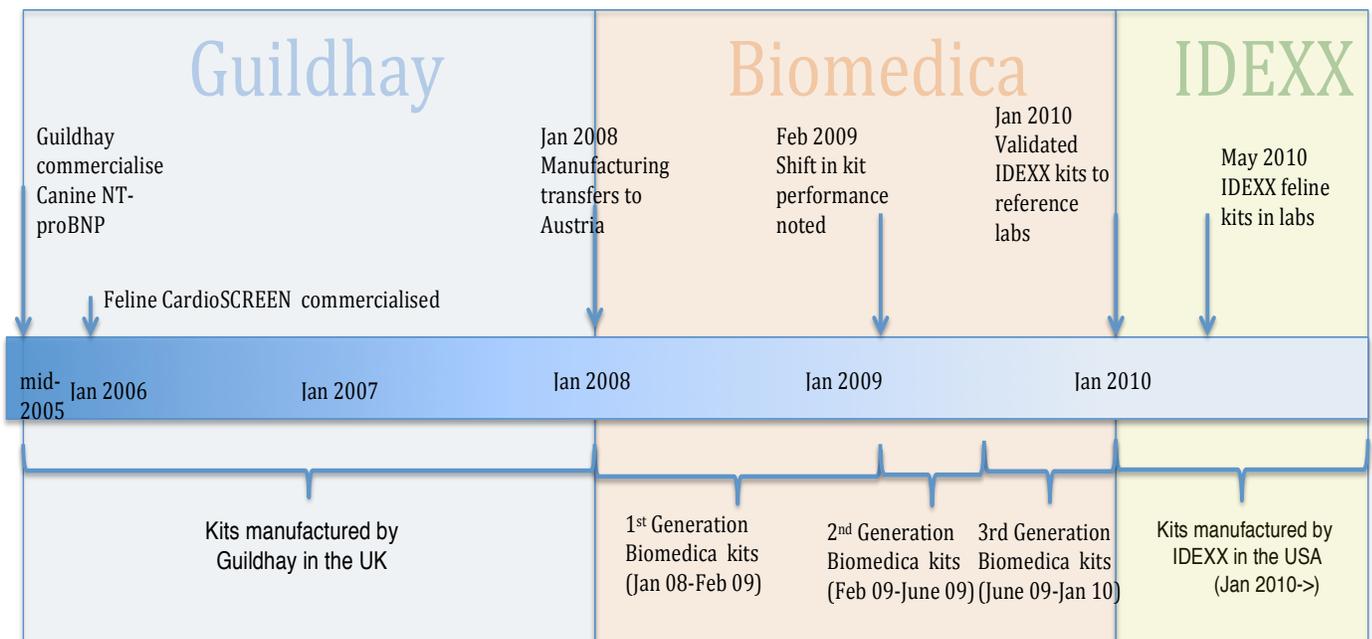
A sandwich enzyme immunoassay designed to measure the immunoreactive NT-proBNP in feline and canine plasma is commercially available. To achieve high specificity the kits incorporate two immunoaffinity purified sheep antibodies specific for feline or canine NT-proBNP. The plate consists a capture antibody, anti-NT-proBNP (25-41) bound to the wells of the plate and a tracer, comprised of detection antibodies, anti-NT-proBNP (1-22) conjugated to horseradish peroxidase. In the incubation step, calibrator or sample and conjugated-detection antibody (conjugate) are added simultaneously to the wells. If present in the sample, NT-proBNP binds to the capture antibody that is precoated in the wells and forms a sandwich with the detection antibody. After a washing step, which removes any nonspecific bound material, substrate is added to the wells. Bound NT-proBNP is quantified by an enzyme-catalyzed color change detectable on a standard ELISA plate reader. The amount of colour developed is directly proportional to the amount of NT-proBNP immunoreactivity present in the calibrator or sample. A calibrator curve is plotted from the values measured, and the concentration of NT proBNP in the samples is calculated from this curve.

Commercial history of feline and canine NT-proBNP assay

The immunoassay for feline and canine NT-proBNP has gone through a number of changes since being commercialised as a diagnostic test (see Figure 1 below).

VetSign™ Canine CardioSCREEN was first manufactured and supplied in 2005 by *Guildhay*¹. *VetSign™ Feline CardioSCREEN Test Kit*, also manufactured by *Guildhay*, became available in 2006. *Veterinary Diagnostics Institute*², marketed the *Guildhay*-manufactured NT-proBNP immunoassay as *CardioCare®* and were exclusive users of the test in the USA. *Biomedica Gruppe*³, the parent company of *Guildhay*, sold the patent on veterinary applications of NT-proBNP to *IDEXX Laboratories*⁴ in 2008. *Guildhay* manufacture of NT-proBNP assay was subsequently stopped in the UK, however *Biomedica* continued to manufacture kits in Austria for *IDEXX* until January 2010. *IDEXX Laboratories* are now the sole worldwide manufacturer and supplier of NT-proBNP assay for cats and dogs.

Figure 1 – Commercial history of feline and canine NT-proBNP assay



¹ *Guildhay Ltd, Unit 6, The Riverside, Business Centre, Walnut Tree Close, Guildford, Surrey, UK.*

² *Veterinary Diagnostics Institute, 4685 Runway Street, Simi Valley, California, USA.*

³ *Biomedica Gruppe Österreich, A-1210 Wien, Divischgasse 4, Austria.*

⁴ *IDEXX Laboratories Ltd., 1 IDEXX Drive, Westbrook, Maine, USA.*

Currently available NT-proBNP assay systems for cats and dogs

IDEXX Laboratories currently manufacture and supply two NT-proBNP testing systems;

- **Cardiopet[®] proBNP**

Exclusive to IDEXX laboratories and used to measure NT-proBNP concentration in K3-ethylenediaminetetraacetic acid (EDTA)-treated plasma (EDTA plasma) that has been shipped in Cardiopet[®] proBNP pink-top transport tubes containing proteinase inhibitors.

- **VetSign[™] Feline and Canine CardioSCREEN Test Kit**

Manufactured and supplied by IDEXX laboratories for commercial use by non-IDEXX laboratories to measure NT-proBNP concentration in EDTA plasma.

Cardiopet[®] and VetSign[™] CardioSCREEN Test Kit are designed to produce the same measured NT-proBNP concentration in a given sample.

Current and historical recommendations for interpretation of NT-proBNP measurements in cats and dogs

The illustrated algorithm in Figure 2(a), produced by IDEXX Laboratories, is recommended for interpretation of measured NT-proBNP concentration in cats and dogs using Cardiopet[®] proBNP. Kit inserts supplied with VetSign[™] Feline and Canine CardioSCREEN Test Kit recommend identical interpretative criteria.

Historically Guildhay, Veterinary Diagnostic Institute and Biomedica have all used cut-off values and algorithms for interpretation of NT-proBNP measurements in dogs, based on clinical studies (Boswood et al., 2008; Oyama et al., 2008; Oyama et al., 2009; Tarnow et al., 2009). However, normal cut-off values have been variable and interpretative algorithms have changed over time (see Figure 2 (b)) as new assay systems have developed and better understanding of sample handling effects have evolved (Beardow and Bilborough, 2010).

Figure 2 (a) - Algorithm supplied by IDEXX for interpretation of measured NT-proBNP concentration in cats and dogs using Cardiopet® proBNP and VetSign™ Feline and Canine CardioSCREEN Test Kit

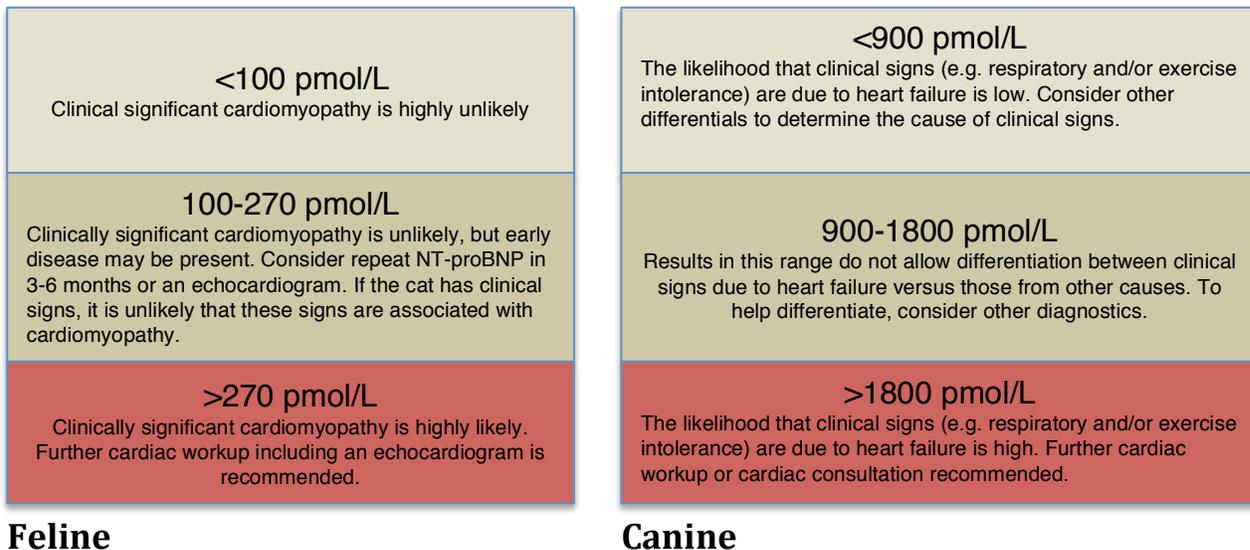
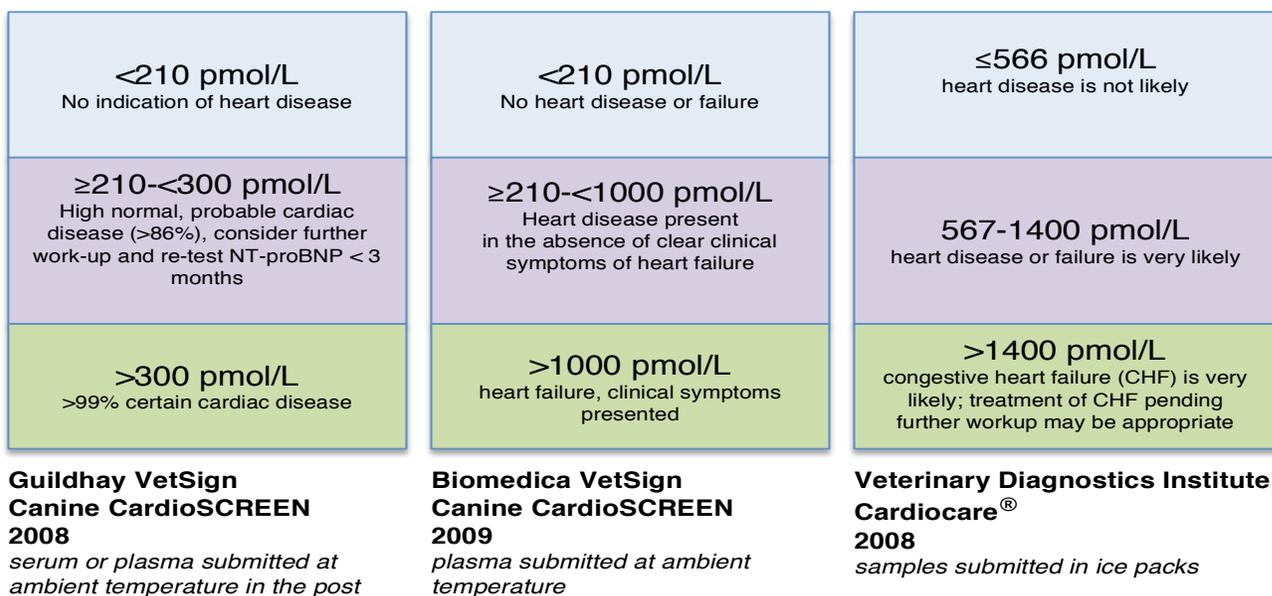


Figure 2 (b) – Historical algorithms supplied by Guilhay, Biomedica and Veterinary Diagnostics Institute (Cardiocare®) for interpretation of measured NT-proBNP in dogs using VetSign™ Canine CardioSCREEN



Historical variation in normal cut-offs

A number of studies have suggested cut-off values for the clinical interpretation of measured NT-proBNP concentration in cats (Connolly et al., 2008; Connolly et al., 2009; Hsu et al., 2009; Ettinger, 2010; Fox et al., 2011; Wess et al., 2011) and dogs (Boswood et al., 2008; Fine et al., 2008; Oyama et al., 2008; Oyama et al., 2009; Tarnow et al., 2009). Although NT-proBNP has been consistently shown to differentiate cats and dogs with heart disease from cats and dogs that were normal or had primary respiratory disease, there has not been broad agreement on the reference ranges and cut-off values used for interpretation in either species (see Figure 2 and Figure 3)

Note: summary of the study findings and sampling protocols tabulated in Appendices.

Figure 3 – Cut-off values for feline NT-proBNP concentration used to discriminate normal from heart disease, and dyspnoea caused by heart failure versus primary respiratory disease (current feline IDEXX algorithm for reference)

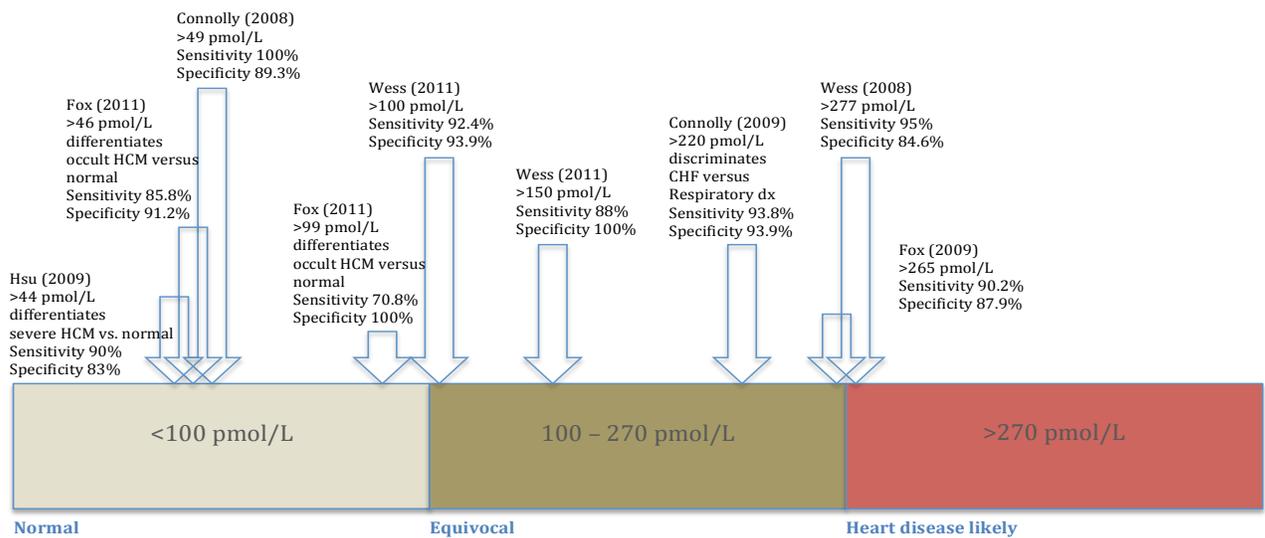
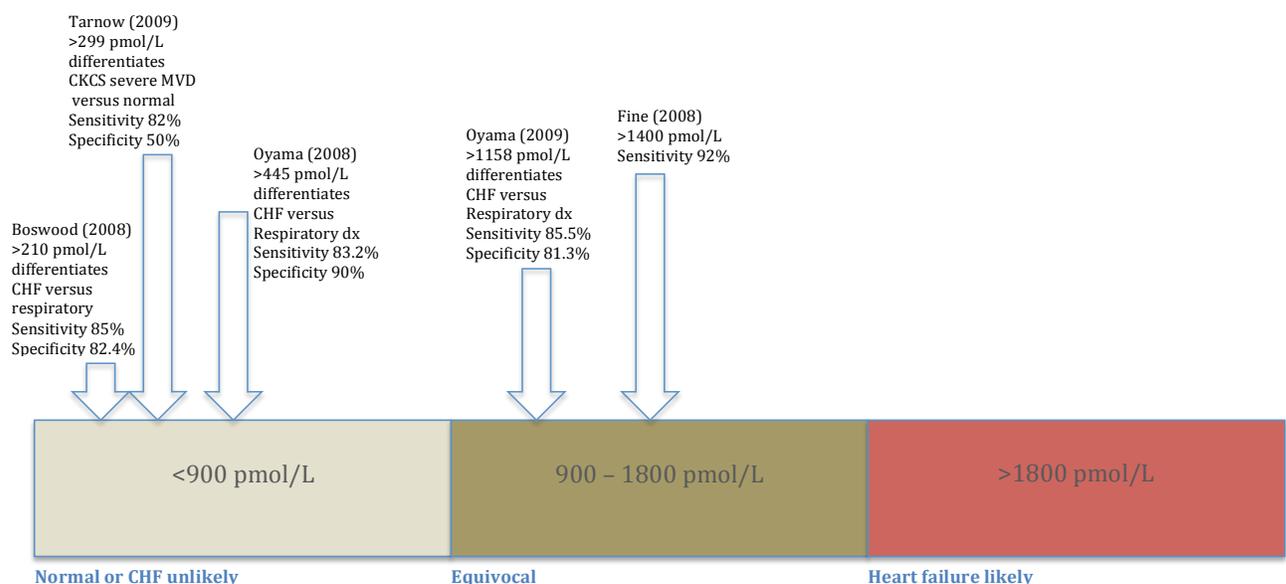


Figure 4 – Cut-off values for canine NT-proBNP concentration used to discriminate normal from heart disease, and dyspnoea caused by heart failure versus primary respiratory disease (current canine IDEXX algorithm for reference)



Obtaining accurate assay results

It is important that blood parameters used for clinical purposes can be accurately measured. The measured substance should be robust enough to withstand standard sampling, processing and transport conditions, or in the case of labile substances, that the sample should be obtained, processed and transported in such a manner that it does not degrade significantly before it reaches the laboratory.

Stability of natriuretic peptides

Humans

In human blood BNP is labile and pre-analytical variability has marked effect on measured concentration, whereas NT-proBNP has been proven to be a relatively robust molecule in various clinical studies, showing good stability in different specimen types and various storage temperatures (Ordonez-Llanos et al., 2008). However, some studies have shown that human NT-proBNP can degrade in serum, heparinised plasma and EDTA plasma, particularly when stored at room temperature. One study showed NT-proBNP in heparinised plasma stored at room temperature decreased by 10% after 2-4 days, 14% after 5 days and 22% after 9 days (Lowbeer & Wallinder, 2007). One hospital study showed unexpectedly low NT-proBNP results in patients with CHF after delayed submission of samples to the laboratory (Lebrun et al., 2007).

Cats

Feline NT-proBNP degrades rapidly at room temperature when stored in EDTA plasma (Connolly et al., 2011). Gunther et al. (2010) showed NT-proBNP, measured within 24 hours of sampling, was similar in EDTA plasma stored at -20°C and EDTA plasma stored in proteinase inhibitor tubes at -20°C and +4°C; however, NT-proBNP was significantly lower when stored in serum at -20°C. Smith (2009) showed poor correlation between measured feline NT-proBNP to the presence and severity of cardiac disease, contrary to published studies (Wess et al., 2008; Connolly et al., 2009; Fox et al., 2009). This was attributed to NT-proBNP degradation during transit to the laboratory.

Dogs

One study showed NT-proBNP degrades rapidly in canine serum, with 50% decrease in NT-proBNP by 24 hours when stored at +25°C (Collins *et al.*, 2010). Another study showed bioreactivity in canine plasma decreased to 50% in 2/3 of samples tested when stored at +25°C (Farace et al., 2008).

Analytical validation of the NT-proBNP assay

Analytical validation of Feline Cardiopet[®] proBNP, using shorter incubation time of 7 hours (original recommended incubation time was 20 hours), showed robust performance with reliable detection limits between 20 and 1500pmol/L (Carrier *et al.*, 2009). VetSign[™] Canine CardioSCREEN manufactured by Guilhay has been validated and shown to perform according to the manufacturer's recommendations (Boswood *et al.*, 2008; Ziebal *et al.*, 2008). Canine Cardiopet[®] proBNP has not been independently validated to the author's knowledge.

Although progressive generations of assay kit have been validated, there have been significant shifts in kit performance associated with changes in assay manufacturer (personal communication, Andy Beardow, IDEXX; Beardow and Bilborough, 2010) (see Figure 1).

Obtaining accurate assay results in practice

Protocol for NT-proBNP measurement using VetSign[™] CardioSCREEN[®] requires that EDTA plasma samples are frozen, transported on ice and arrive at the laboratory frozen. Although general practitioners are accustomed to shipping frozen samples on ice, the process is time consuming and often performed in a sub-optimal fashion. Furthermore protocol requires that samples that have thawed in transit should be discarded, necessitating further sample collection; a significant inconvenience for the animal, owner and clinician involved.

In order to improve the clinical utility of NT-proBNP measurement in practice, a transport tube (Cardiopet[®] proBNP pink-top transport tubes) containing protease inhibitor was developed by IDEXX laboratories, allowing shipping of samples at ambient temperature. Protocol requires that whole blood is placed in an EDTA tube, immediately centrifuged at 1500g, plasma separated, placed in Cardiopet[®] proBNP pink-top transport tubes and shipped at ambient temperature to arrive at the laboratory within 24 hours of sampling. Although this system is less laborious than shipping samples on ice, it requires non-standard sample collection and use of specialist transport tubes. Furthermore the Cardiopet[®] proBNP pink-top transport tubes are patented by IDEXX, and therefore testing of proteinase-inhibited EDTA plasma is only offered through IDEXX laboratories.

Reason for the study - Limitations for NT-proBNP use in practice

- NT-proBNP is marketed to general veterinary practitioners as a screening test for feline and canine heart disease and heart failure; therefore it is important that the test performs reliably when used in typical practice conditions. Despite practical limitations to routine use of NT-proBNP testing in small animal practice, the assay is increasingly utilised as practitioners become aware of its existence and realise its potential diagnostic value.
- NT-proBNP studies in cats and dogs have largely been performed in research institutions where sample-handling protocols have been rigorous (see Tables A – F in appendices). Although numerous studies have shown promising clinical utility, none have been performed in general practice conditions where it is likely that sample-handling protocols will be less rigorously adhered to. NT-proBNP in canine serum and plasma shows temporal and temperate instability. In order to determine how this might affect measuring NT-proBNP in practice, a study is required to quantify the effect of time and storage temperature on both feline and canine NT-proBNP in serum and plasma stored and processed in typical small animal practice conditions.
- Sampling protocol for NT-proBNP assay requires centrifugation, separation and storage of plasma within 60 minutes of sample collection, prohibiting use of samples that cannot be processed immediately. Blood samples are often obtained at branch surgeries without centrifugation facilities and in many situations in general small animal practice samples may not be processed for several hours after collection. NT-proBNP concentration in plasma obtained from whole blood processed later than 60 minutes after collection has not been studied.
- A number of commercial laboratories offer VetSign™ Feline and Canine CardioSCREEN NT-proBNP assay in frozen EDTA plasma. Samples shipped to the laboratory on ice must be discarded if they thaw in transit. NT-proBNP stability in frozen EDTA plasma transported in field conditions to laboratories using VetSign™ Feline and Canine CardioSCREEN has not been studied. Given that NT-proBNP levels remain stable for 24 hours when stored at -4°C (Farace et al., 2008), current recommendations to discard samples that have thawed in transit may necessitate disposal of perfectly usable samples.
- Cardiopet® proBNP (IDEXX) used in conjunction with patented proteinase inhibitor tubes was developed to reduce sample degradation in transit. Although one study showed reduced degradation in proteinase inhibited feline plasma (Connolly et al., 2011), a study comparing NT-proBNP measured using Cardiopet® proBNP versus CardioSCREEN® in practice conditions has not been performed.

Study aims

The aim of this study was to determine if, and to what degree, sample preparation and shipping methods commonly employed in general veterinary practice might affect measured concentration of feline and canine NT-proBNP.

Specific aims

To determine if, and to what degree, temporal and temperate degradation of feline and canine NT-proBNP might occur in;

- *Serum and EDTA plasma stored at room temperature (+25⁰C) and refrigerator temperature (+4⁰C) over 72 hours*
- *EDTA plasma processed from whole blood which had been stored at room temperature (+25⁰C) and refrigerator temperature (+4⁰C) over 9 hours*
- *EDTA plasma frozen at -20⁰C and then stored on ice at ambient temperature over 96 hours.*

To determine if, and to what degree, feline and canine NT-proBNP concentration might differ in

- *protease-inhibited EDTA plasma, shipped by courier and measured using Cardiopet[®] proBNP versus*
- *frozen EDTA plasma, measured by another commercial laboratory using VetSign[™] CardioSCREEN Test Kit*

To determine if, and to what degree, feline and canine NT-proBNP concentration might be affected by;

- *contact with separator gel in serum*
- *multiple freeze thaw cycles*

MATERIALS and METHODS

Study population

Blood samples were obtained from cats and dogs with evidence of heart disease presented for diagnostic investigation at the author's clinic⁵.

Blood samples were obtained only when required for clinical purposes and residual sample was used for study assays. Owner consent for blood sampling, and for the use of residual sample for research purposes, was obtained in all cases.

All animals received thorough physical and echocardiographic examination. In most cases haematology, biochemistry, electrocardiography, thoracic radiography and blood pressure measurement were also performed.

Sample collection

All blood samples were obtained by jugular venipuncture and collected into plastic syringes. Sample volume varied according to body weight and varied from 2.5 to 7.5mls. Blood not required for haematological and biochemical analysis was transferred into 1.3ml plastic blood collection tubes containing EDTA (EDTA tubes), 1.3ml plastic collection tubes without additive (plain tubes) and/or plastic collection tubes with separator gel (gel tubes) depending on the volume available. Sample collection tubes were filled to the marked fill-line in all cases.

Sample preparation

Serum and serum gel

Blood samples in plain tubes and gel tubes, were allowed to clot then centrifuged immediately.

Centrifugation was performed at room temperature at a speed of 1500g for 5 minutes.

Serum in plain tubes (serum) was immediately separated and transferred to plain polypropylene (Eppendorf) tubes for storage. Serum in gel tubes (serum gel) was not separated and stored in contact with the separator gel.

EDTA plasma

Blood samples placed in 1.3ml EDTA tubes were centrifuged at room temperature at a speed of 1500g for 5 minutes immediately after collection. EDTA plasma was immediately separated and stored in plain Eppendorf tubes.

⁵ Southern Counties Veterinary Specialists, Unit 6, Forest Corner Farm, Hangersley, Ringwood, Hampshire.

EDTA plasma_{PI} (EDTA plasma with added proteinase inhibitor)

EDTA plasma_{PI} was prepared by placing an aliquot of 0.3ml – 1.0ml EDTA plasma in IDEXX Pink-Top proteinase inhibitor tubes within 30 minutes of collection, according to laboratory protocol.

All EDTA plasma_{PI} samples were dispatched strictly according to laboratory recommendations; samples were transported by courier at ambient temperature to arrive at the laboratory within 24 hours; thereafter samples were analysed immediately or snap frozen at -80°C for analysis at a later date. Samples obtained outside normal courier working hours (and therefore unlikely to arrive at the laboratory within 24 hours) were stored at -20°C and dispatched at a later date, but not longer than 20 days for any sample.

Sample storage

Samples were stored at room temperature (+25°C), refrigerated (+4°C), frozen (-20°C) or packaged in ice-packs, according to study protocols described below. Room temperature, refrigerator and freezer temperature was recorded once daily during the study period.

NT-proBNP Assay

Plasma and serum NT-proBNP was measured using VetSign™ Canine or Feline Cardioscreen Test Kit at an on-site commercial veterinary laboratory⁶ The kit was used according to the manufacturer's instructions. Incubation time was 5 hours for feline samples and 18 hours for dogs. Photometry was performed using a Biotek Elx 800 microtitre photometer plate reader.

Five calibration samples and three control samples (see Table 1) with known NT-proBNP concentration were measured in duplicate for each test plate (96 wells), according to manufacturer's instructions.

Table 1 – Target values (and range) for feline and canine control samples

Control samples	Low concentration	Medium concentration	High concentration
Feline	230 pmol/L(± 72)	541 pmol/L (± 180)	1236 pmol/L (± 524)
Canine	477 pmol/L(±131)	1002 pmol/L (±202)	1662 pmol/L (± 312)

Intra-assay coefficient of variation was calculated for calibrator and control samples.

NT-proBNP in EDTA plasma_{PI} was measured using Cardiopet® proBNP by IDEXX Laboratories⁷.

⁶ *Torrance Diamond Diagnostic Services - Ringwood (TDDS Ringwood), Unit 2c, Forest Corner Farm, Hangersley, Ringwood, Hampshire, UK*

⁷ *IDEXX Laboratories, Grange House, Sandbeck Way, Wetherby, West Yorkshire, UK*

Statistics

All statistical analyses were undertaken with standard statistical software (SPSS version 20.0). Quantitative data were assessed for normality graphically and were described with median and interquartile range. The Friedman's two-way analysis of variance by ranks was used to compare repeated measurements of NT-proBNP concentrations over time. Statistical significance was set at alpha 0.05. Wilcoxon signed-rank test was also used for *post hoc* comparisons of paired samples. Statistical significance was adjusted to alpha 0.01 to allow for multiple comparisons. Wilcoxon signed-ranks test was also used for comparison of paired samples (studies D and E). Statistical significance was set at alpha 0.05.

Six studies were performed as follows;

Study A - Temporal effect on feline and canine NT-proBNP concentration in EDTA plasma and serum stored at +4°C and +25°C for 72 hours.

Study B - Temporal effect on feline and canine NT-proBNP concentration in whole blood stored in EDTA tubes at +4°C and +25°C for 9 hours

Study C - Temporal effect on feline and canine NT-proBNP concentration in frozen EDTA plasma stored in ice packs at room temperature (+25°C) for 96 hours.

Study D - Comparison of feline and canine NT-proBNP concentration in protease inhibited EDTA plasma measured using Cardiopet®proBNP versus NT-proBNP concentration in frozen EDTA plasma measured using VetSign™ CardioSCREEN Test Kit

Study E - Effect of separator gel on feline and canine serum NT-proBNP concentration

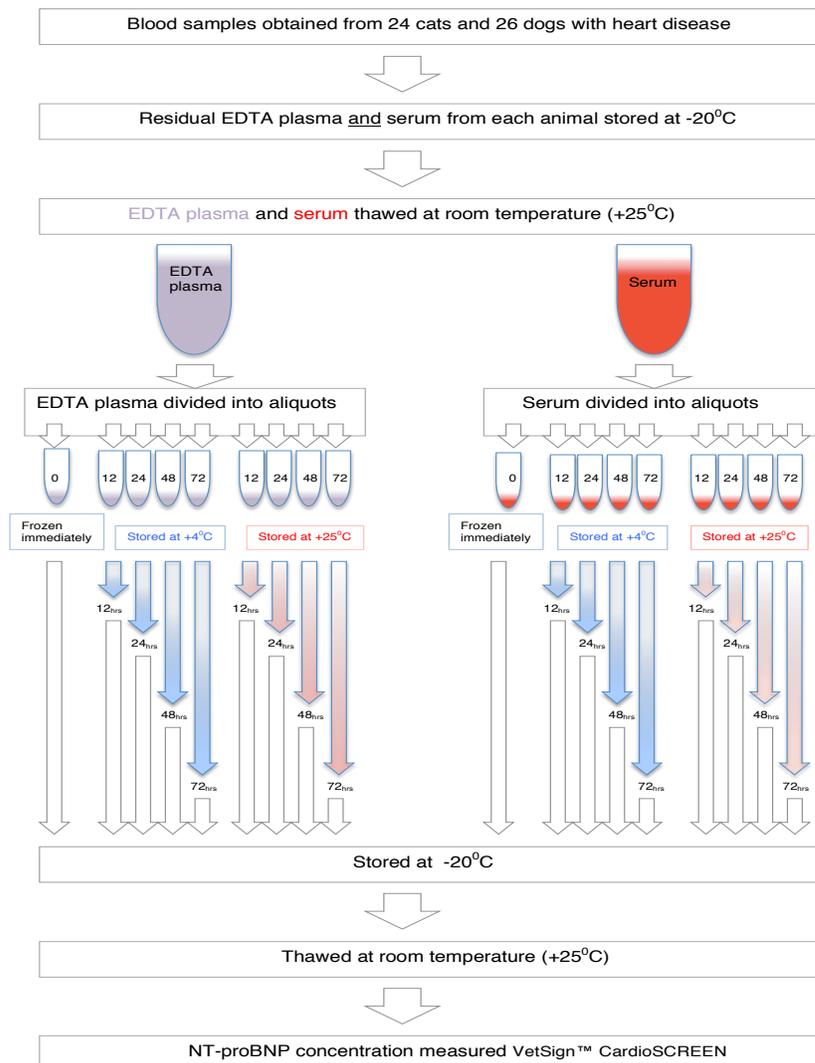
Study F - Effect of multiple freeze thaw cycles on feline and canine NT-proBNP concentration

Study A - Temporal effect on feline and canine NT-proBNP concentration in EDTA plasma and serum stored at +4°C and +25°C for 72 hours.

Method

Residual EDTA plasma and serum was retained from 24 cats and 26 dogs with heart disease then frozen for analysis at a later date (not later 6 months after collection). Samples were allowed to thaw at room temperature. Plasma and serum samples were each divided into nine aliquots of 35-100 µl stored in plain Eppendorf tubes. One aliquot of serum and one aliquot of plasma were immediately re-frozen. The remaining aliquots of plasma and serum were divided into two equal batches and stored at either +4°C or +25°C. One plasma and one serum sample from each batch was re-frozen 12, 24, 48 and 72 hours later. All samples were thawed at room temperature 7 – 30 days later and NT-proBNP was measured.

Figure 5 – Method for study A - Temporal effect on feline and canine NT-proBNP concentration in EDTA plasma and serum stored at +4°C and +25°C for 72 hours.

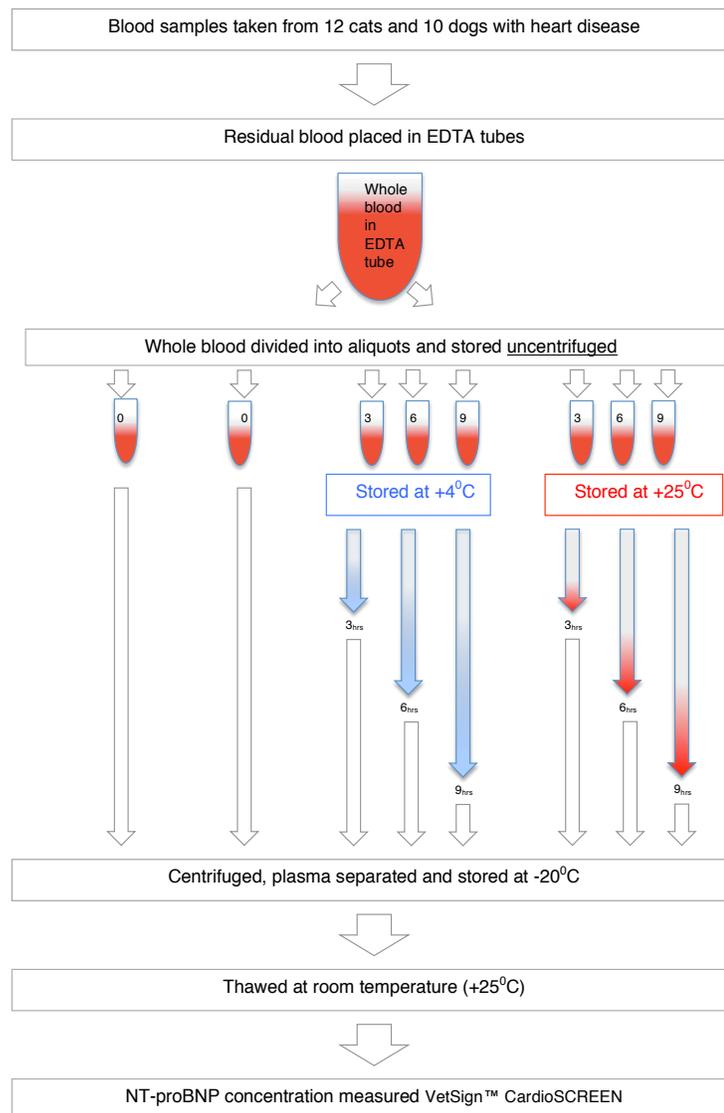


Study B - Temporal effect on feline and canine NT-proBNP concentration in whole blood stored in EDTA tubes at +4°C and +25°C for 9 hours

Method

Residual blood obtained from 12 cats and 10 dogs with heart disease was placed in EDTA tubes. Samples were divided into eight aliquots of whole blood. Two aliquots were immediately centrifuged, plasma separated and frozen at -20°C. The remaining six aliquots of whole blood were divided into two batches and stored at either +4°C or +25°C. At 3 hours, 6 hours and 9 hours after sample collection, one aliquot from each batch was immediately centrifuged, plasma separated and frozen at -20°C. All samples were thawed at room temperature 7 – 30 days later and NT-proBNP was measured.

Figure 6 – Method for study B - Temporal effect on feline and canine NT-proBNP concentration in whole blood stored in EDTA tubes at +4°C and +25°C for 9 hours

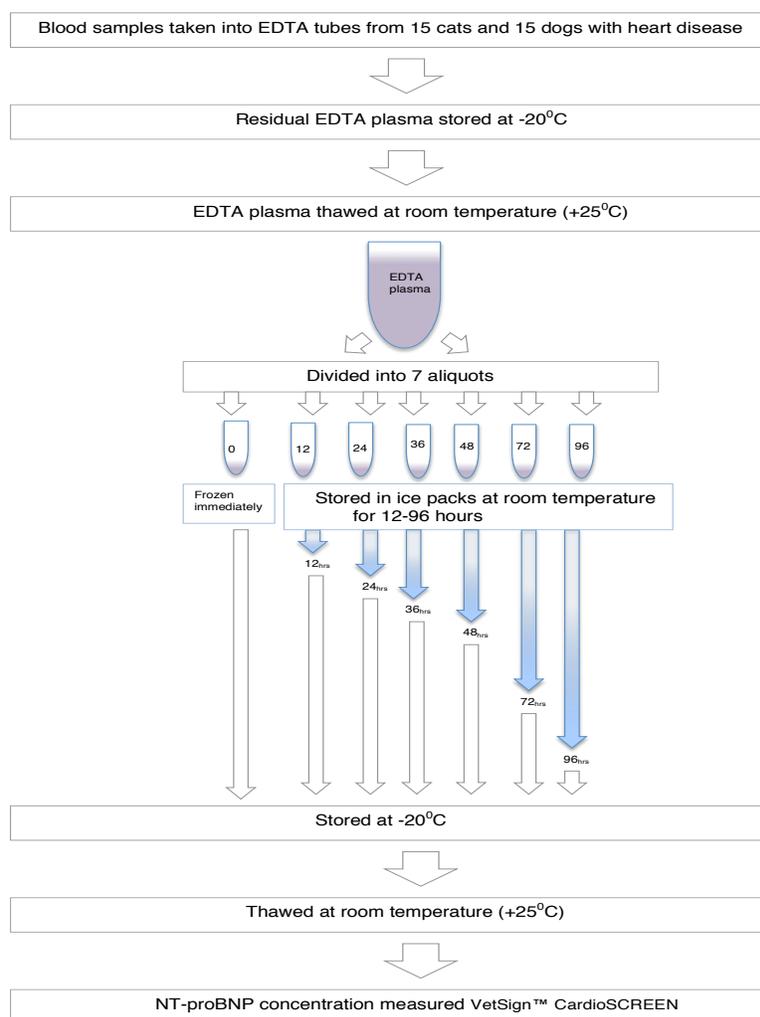


Study C - Temporal effect on feline and canine NT-proBNP concentration in frozen EDTA plasma stored in ice packs at room temperature (+25°C) for 96 hours.

Method

Residual EDTA plasma from 15 cats and 15 dogs with heart disease was stored at -20°C for up to 30 days. Samples were thawed at room temperature, immediately divided into seven aliquots stored in plain Eppendorf tubes and re-frozen. One aliquot from each animal was stored at -20°C and the remaining six aliquots from each animal were enclosed in ice-packs. Ice-packs were prepared by enveloping samples in frozen ice bags, which were then packed inside a polythene bag and padded paper postage bag. Packages were stored at room temperature. One batch of samples was removed from its packaging and re-frozen at -20°C for analysis at a later date 12, 24, 36, 48, 72 and 96 hours later. All samples were thawed at room temperature 7 – 30 days later and NT-proBNP was measured.

Figure 7 – Method for study C - Temporal effect on feline and canine NT-proBNP in frozen EDTA plasma stored in ice packs at +25°C for 96 hours.

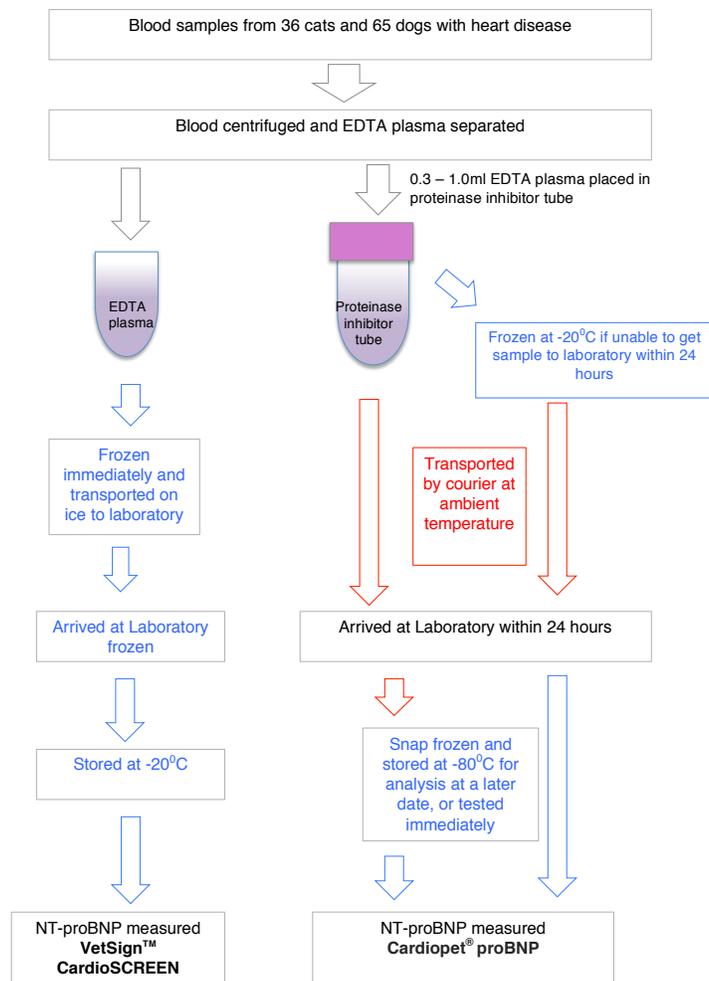


Study D - Comparison of feline and canine NT-proBNP concentration in protease inhibited EDTA plasma measured using Cardiopet[®] proBNP versus NT-proBNP concentration in frozen EDTA plasma measured using VetSign[™] CardioSCREEN

Method

Blood samples were obtained from 36 cats and 65 dogs for NT-proBNP assay. EDTA plasma_{PI} was prepared and shipped as described above. EDTA plasma was delivered immediately to the on-site laboratory where it was stored at -20⁰C for analysis at a later date, not longer than 30 days (cats) or 6 months (dogs) later. NT-proBNP in EDTA plasma_{PI} was measured using Feline or Canine Cardiopet[®] proBNP and was compared with NT-proBNP in EDTA plasma measured using VetSign[™] Feline or Canine Cardioscreen using Wilcoxon signed-ranks (alpha 0.05).

Figure 8 – Method for study D - Comparison of feline and canine NT-proBNP in protease inhibited EDTA plasma measured using Cardiopet[®] proBNP versus NT-proBNP in frozen EDTA plasma measured using VetSign[™] CardioSCREEN

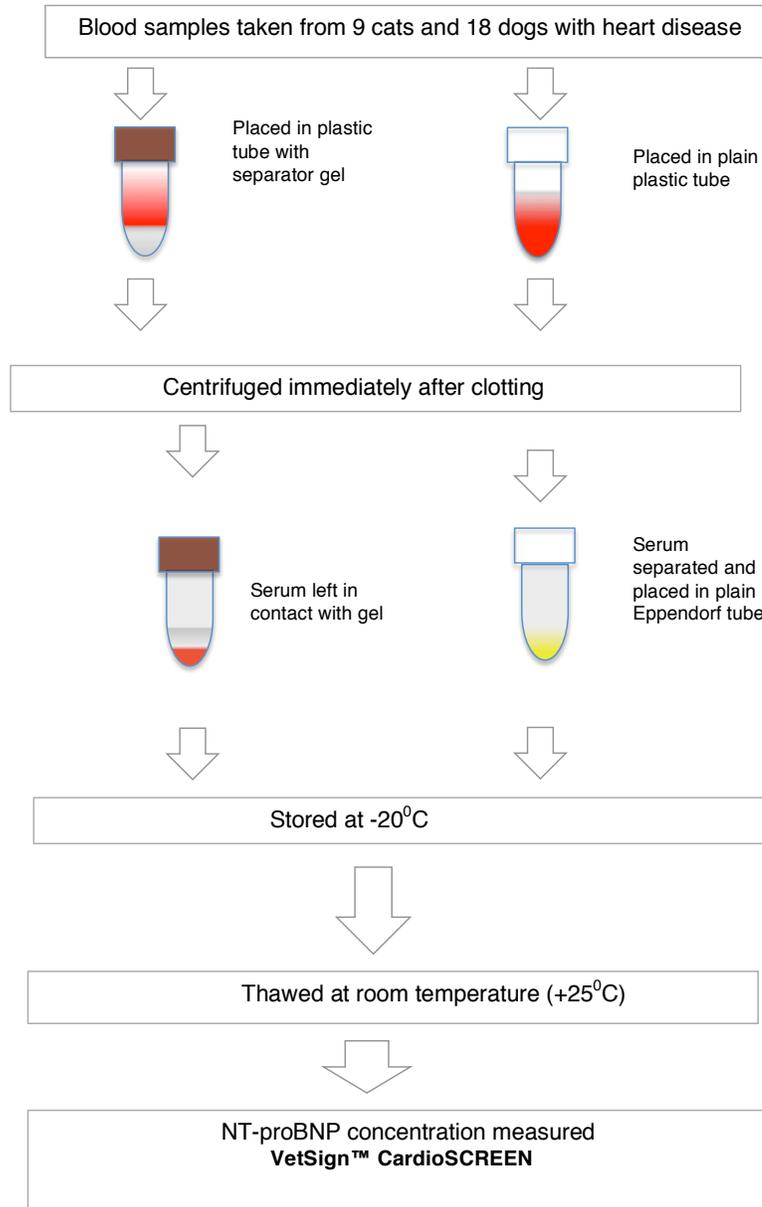


Study E - Effect of separator gel on feline and canine serum NT-proBNP concentration

Method

Residual serum samples from 9 cats and 18 dogs were placed in plain tubes and separator gel tubes then stored at -20°C for up to 6 months. Samples were thawed and NT-proBNP was measured. Results were compared using Wilcoxon signed-rank test (alpha 0.05).

Figure 9 – Method for study E - Effect of separator gel on feline and canine serum NT-proBNP concentration

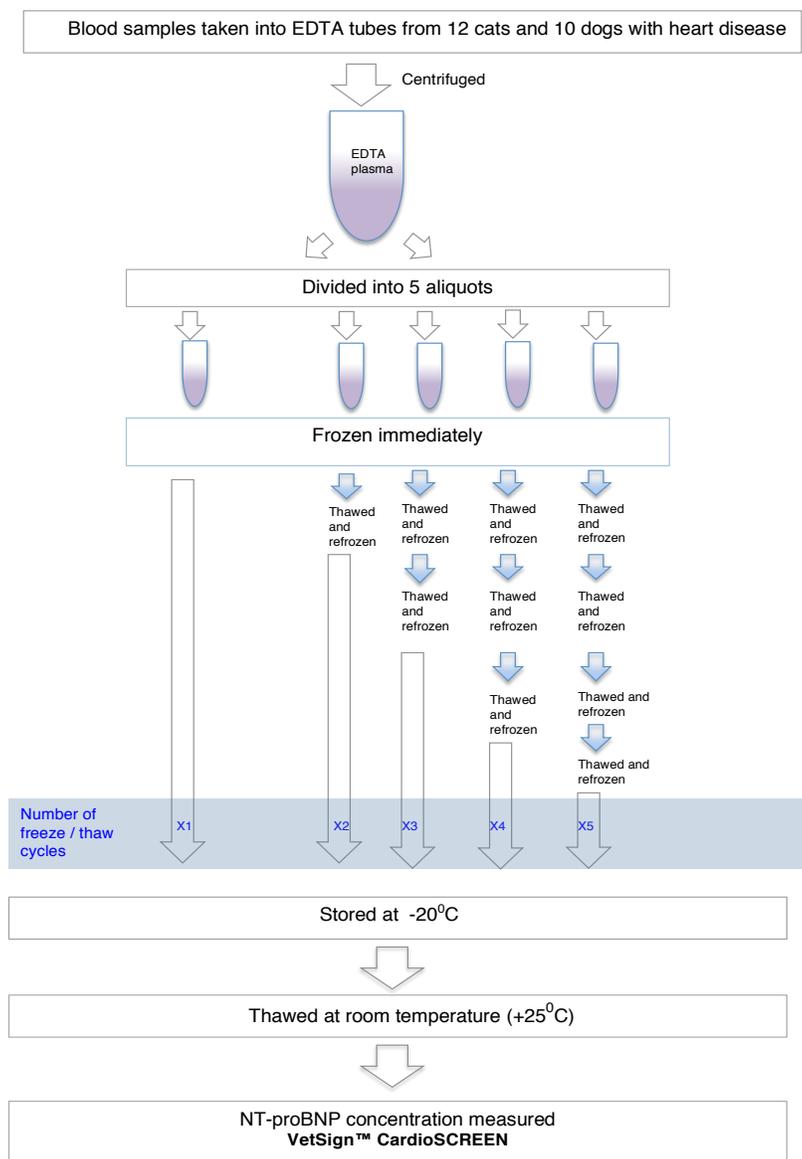


Study F -Effect of multiple freeze thaw cycles on feline and canine EDTA plasma NT-proBNP concentration

Method

Residual EDTA plasma from 12 cats and 10 dogs was divided into five aliquots and stored at -20°C. Four aliquots were allowed to thaw at room temperature. NT-proBNP was measured in one aliquot and remaining aliquots were re-frozen. The process was repeated three times. Samples were re-frozen at -20°C for a minimum of 24 hours between each freeze-thaw cycle. NT-proBNP concentration was compared after one, two, three, four and five freeze/thaw cycles.

Figure 10 – Method for study F - Effect of multiple freeze thaw cycles on feline and canine EDTA plasma NT-proBNP concentration



RESULTS

Animals

NT-proBNP was measured in a total of 55 cats and 70 dogs.

Cat population

Mean (\pm SD) age was 7.8 years (\pm 4.1); age range was 10 months to 16.8 years. There were 35 males and 20 females.

Six breed types were represented; Domestic Shorthair (n=38), Domestic Longhair (n=5), British Shorthair (n=4), Siamese (n=4), Maine Coon (n=2) and British Blue (n=2).

Hypertrophic cardiomyopathies were most commonly diagnosed (n=44); sub-classified to hypertrophic obstructive cardiomyopathy (n=20), hypertrophic cardiomyopathy (n=16) and end-stage hypertrophic cardiomyopathy (n=8). Two cats had unclassified cardiomyopathy, two cats (both Siamese) had feline endomyocardial restrictive cardiomyopathy and four cats had left ventricular hypertrophy secondary to systemic disease. One cat had severe aortic insufficiency, one cat had septic pericarditis and one cat had idiopathic chylothorax.

Dog population

Mean (\pm SD) age was 7.9 years (\pm 3.6); age range was 6 months to 14.3 years.

There were 37 males and 33 females.

There were 28 breed types represented; boxers (n=11), Cavalier King Charles spaniels (n=11), lurcher (n=5), crossbreeds (n=5), Cocker spaniels (n=3), Golden Retrievers (n=3), Border collies (n=3), Irish Wolfhounds (n=3), two each of Bull Mastiff, Dogue de Bordeaux, German Shepherd dog, Great Dane, Labradors and Shih Tzu, and one each of Basset, Beagle, Cairn terrier, Doberman pinscher, English Springer spaniel, Irish Setter, Miniature Schnauzer, Newfoundland, Old English Sheepdog, Rottweiler, Sealyham terrier, Shetland sheepdog, Standard Poodle and West Highland White terrier.

Diagnoses included degenerative mitral valve disease (n=24), dilated cardiomyopathy (n=13), aortic stenosis (n=8), arrhythmogenic right ventricular cardiomyopathy (n=4), cardiac neoplasia (n=2), 3rd degree heart block (n=2), patent ductus arteriosus (n=1), pulmonic stenosis (n=1), tetralogy of Fallot (n=1). Nine dogs were normal or had equivocal evidence of heart disease. Two dogs had pulmonary neoplasia, two dogs had primary pulmonary hypertension and one dog had adrenal neoplasia.

Sample storage

Mean room temperature was 24.8^oC (range 18.6^oC to 28.7^oC). Mean refrigerator temperature was 4.4^oC (range 4.0^oC to 6.8^oC). Mean freezer temperature was -19.9^oC (range -18.4^oC to -20.6^oC).

Assay

Mean laboratory temperature was 22.4⁰C (range 21.9⁰C-23.5⁰C). The upper detection limits were 1500 pmol/L for cats and 3000 pmol/L for dogs.

Mean (\pm SD) inter-assay coefficient of variability for feline and canine calibrators and control samples is illustrated in Table 2 below.

Table 2 - Coefficient of variability for feline and canine calibration and control samples

	Calibrator samples	Mean (\pm SD) Concentration control sample	Low concentration control sample	Medium concentration control sample	High concentration control sample
Feline	12.0% (\pm 3.6%)	17% (\pm 2.7%)	18%	19%	14%
Canine	12% (\pm 5.2%)	15% (\pm 5.9%)	20%,	17%	9%

Study A - Temporal effect on feline and canine NT-proBNP concentration in EDTA plasma and serum stored at +4°C and +25°C for 72 hours.

Cats

Temporal effect - NT-proBNP in feline plasma (see Figure 11) and serum (see Figure 12) stored at +4°C and +25°C decreased over time when compared to baseline measurement at 0 hours ($p < 0.0001$). NT-proBNP in feline plasma (see Figure 11) and serum (see Figure 12) was lower when stored at +25°C when compared to +4°C at all time points after 0 hours ($\alpha 0.01$), except for 12 hours in plasma ($p = 0.673$).

Figure 11 - Change in NT-proBNP concentration in FELINE PLASMA stored at +4C and +25C over 72 hours

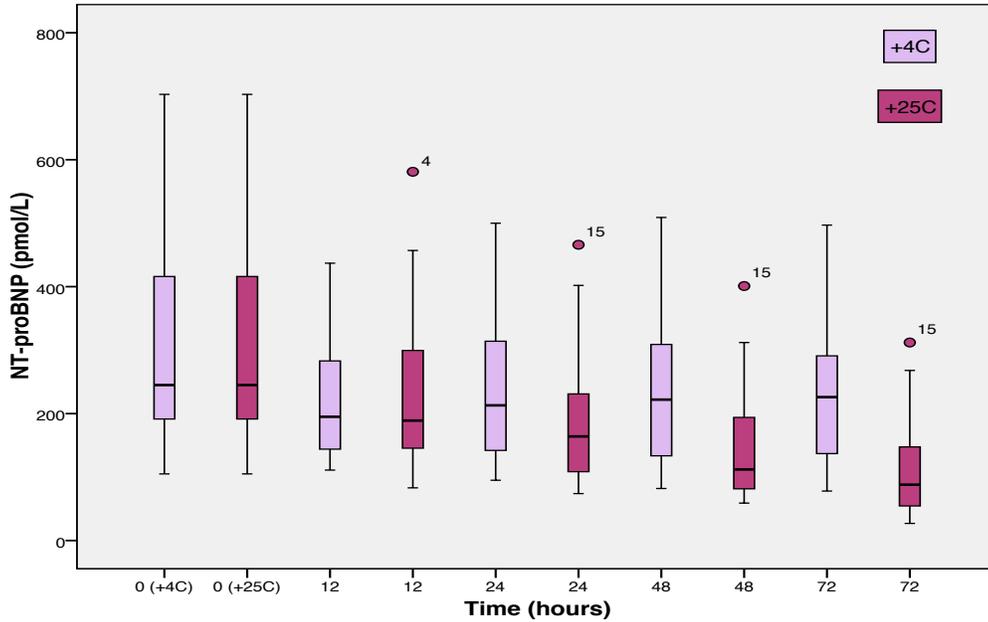
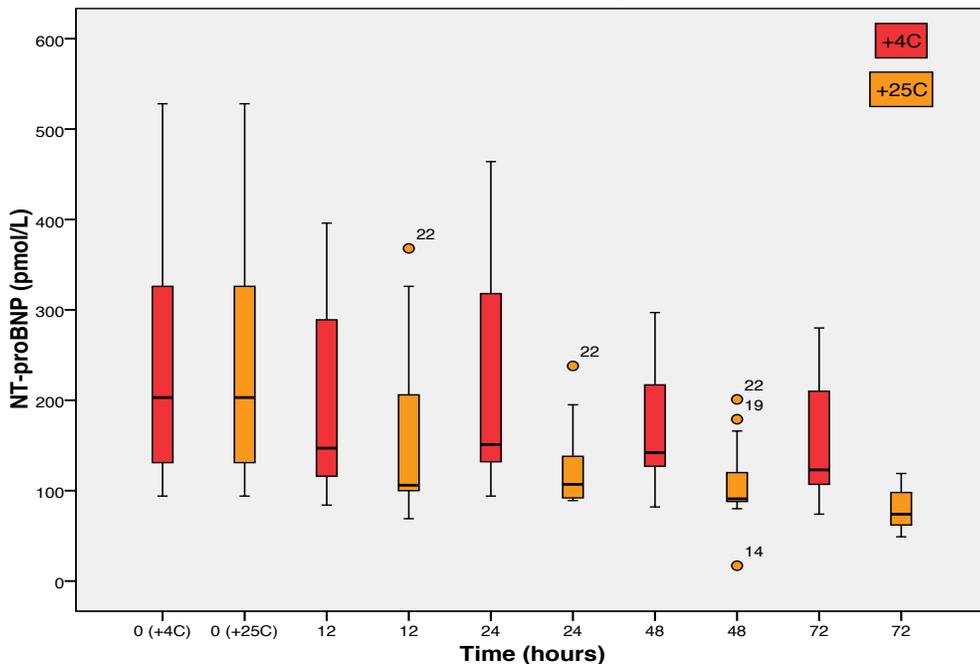


Figure 12 - Change in NT-proBNP concentration in FELINE SERUM stored at +4C and +25C over 72 hours



EDTA plasma versus serum - NT-proBNP was higher in feline plasma compared to serum stored at +4°C (see Figure 13) and +25°C (see Figure 14) at all time points ($p < 0.05$), but failed to reach statistical significance when adjusted for multiple comparisons (alpha 0.01) at 12 hours ($p = 0.055$) and 24 hours ($p = 0.086$) when stored at +4°C, also 24 hours ($p = 0.049$), 48 hours ($p = 0.062$) and 72 hours ($p = 0.182$) then stored at +25°C.

Figure 13 - Change in NT-proBNP concentration in FELINE PLASMA and SERUM stored at +4°C over 72 hours

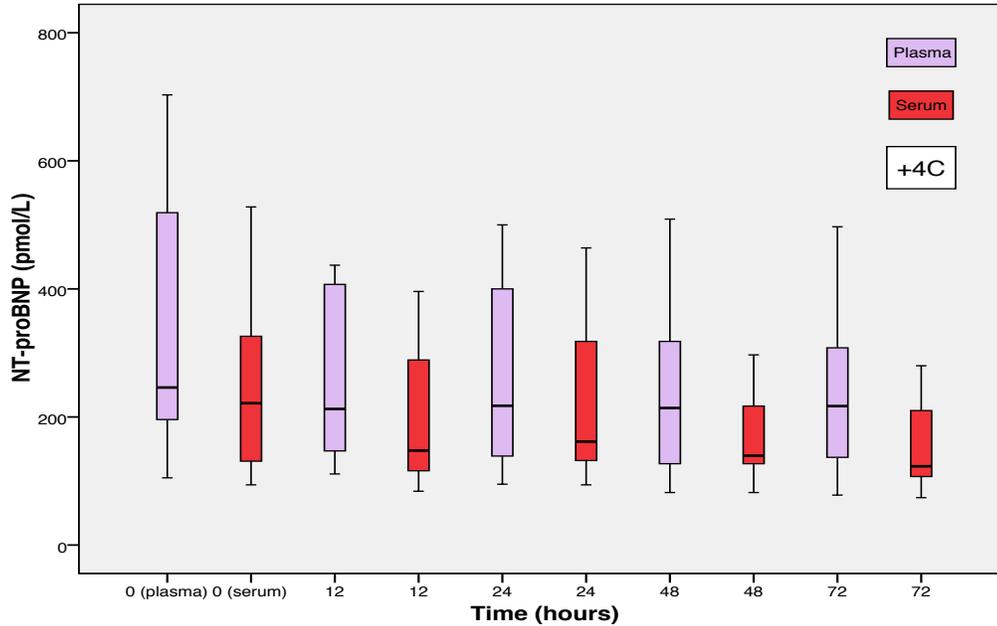
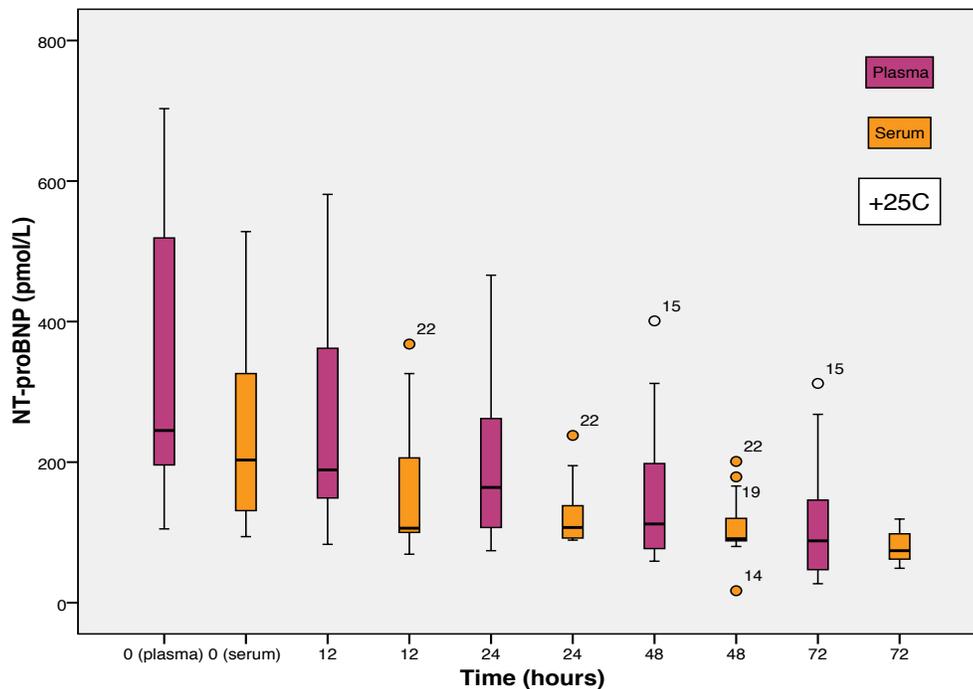


Figure 14 - Change in NT-proBNP concentration in FELINE PLASMA and SERUM stored at +25°C over 72 hours



Dogs

Temporal effect - NT-proBNP in canine plasma (see Figure 15) and serum (see Figure 16) stored at +4°C and +25°C decreased over time when compared to baseline measurement at 0 hours ($p < 0.0001$). NT-proBNP in canine plasma (see Figure 15) and serum (see Figure 16) was lower when stored at +25°C compared to +4°C at all time points after 0 hours ($p < 0.01$), except for 24 hours in plasma ($p = 0.979$).

Figure 15 - Change in NT-proBNP concentration in CANINE PLASMA at +4C and +25C over 72 hours

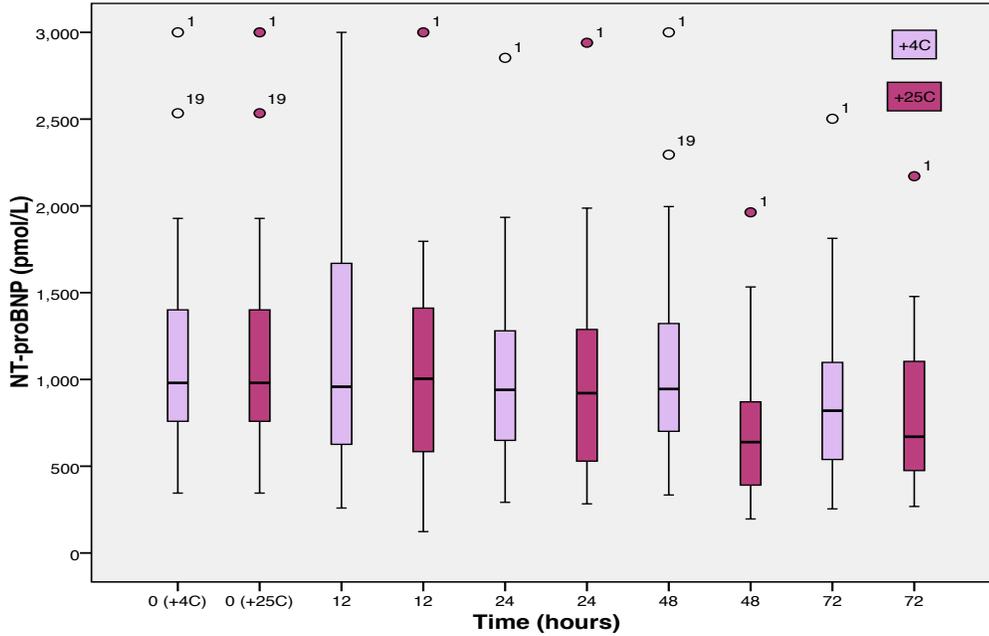
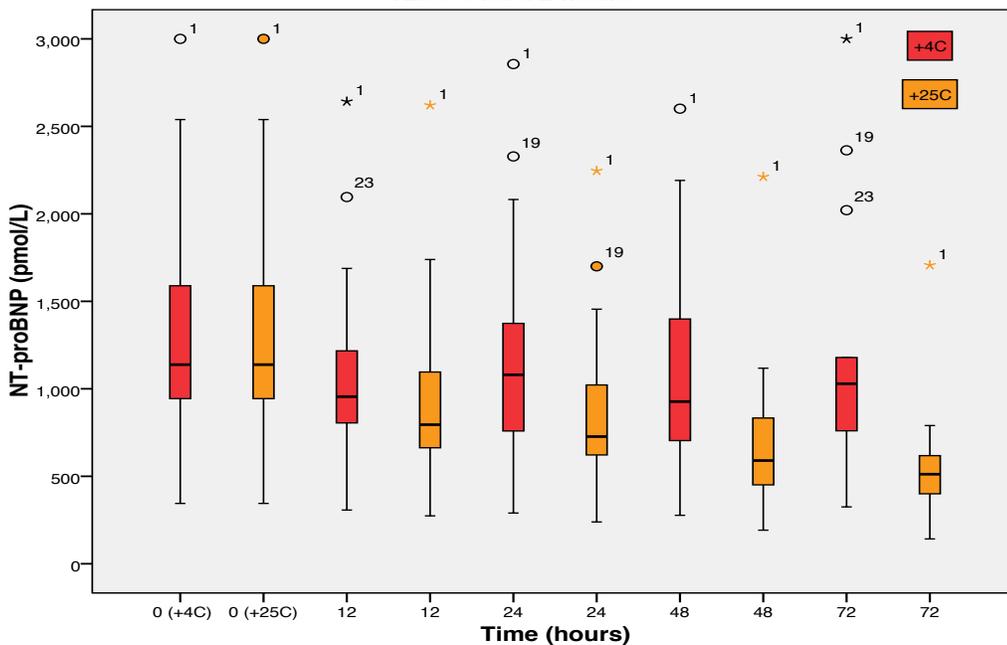


Figure 16 - Change in NT-proBNP concentration in CANINE SERUM at +4C and +25C over 72 hours



EDTA plasma versus serum - NT-proBNP was higher in canine plasma compared to serum when stored at +4°C (see Figure 17) and +25°C (see Figure 18) at all time points (p=0.006), but failed to reach statistical significance when adjusted for multiple comparisons (alpha 0.01) when stored at +4°C at 0 hours (p=0.03), 12 hours (p=0.015), 24 hours (p=0.03), 48 hours (p=0.05) and 72 hours (p=0.03); also when stored at +25°C at 0 hours (p=0.03) and 48 hours (p=0.351).

Figure 17 - Change in NT-proBNP concentration in CANINE PLASMA and SERUM stored at +4C over 72 hours

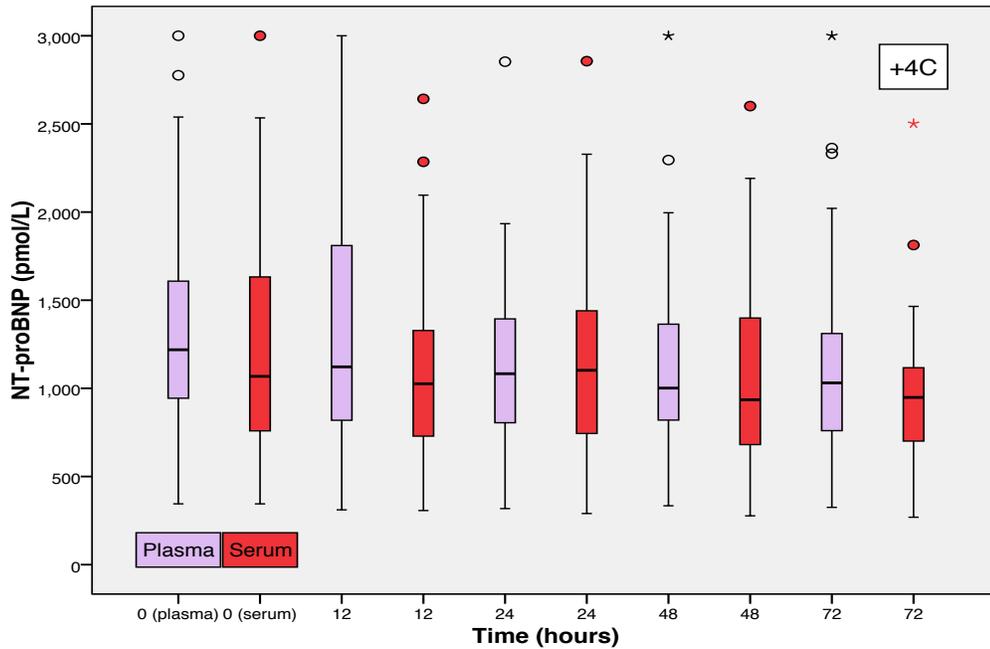
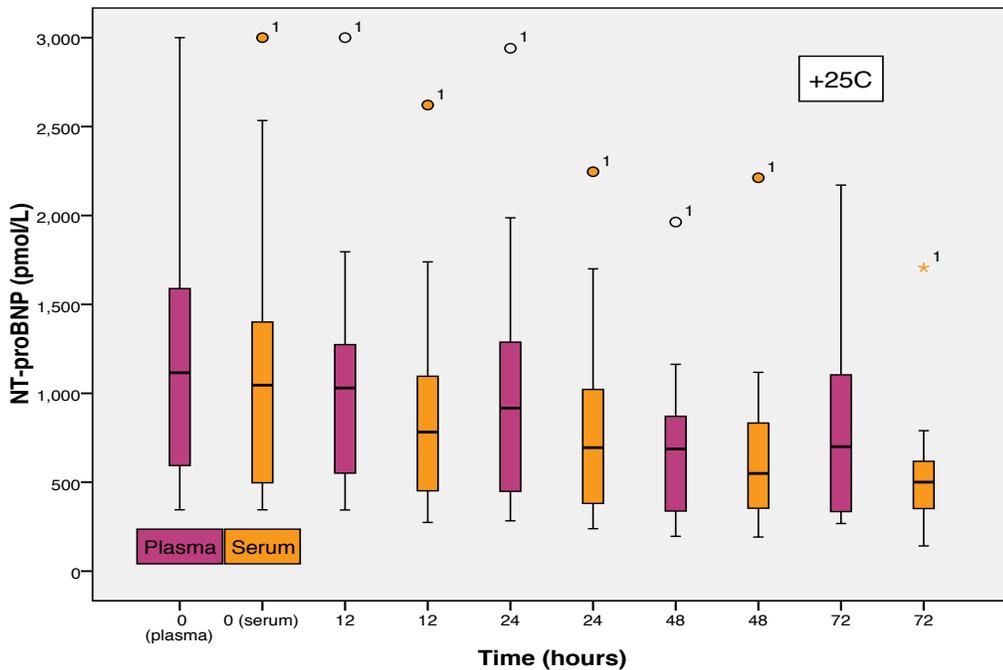


Figure 18 - Change in NT-proBNP concentration in CANINE PLASMA and SERUM stored at +25C over 72 hours

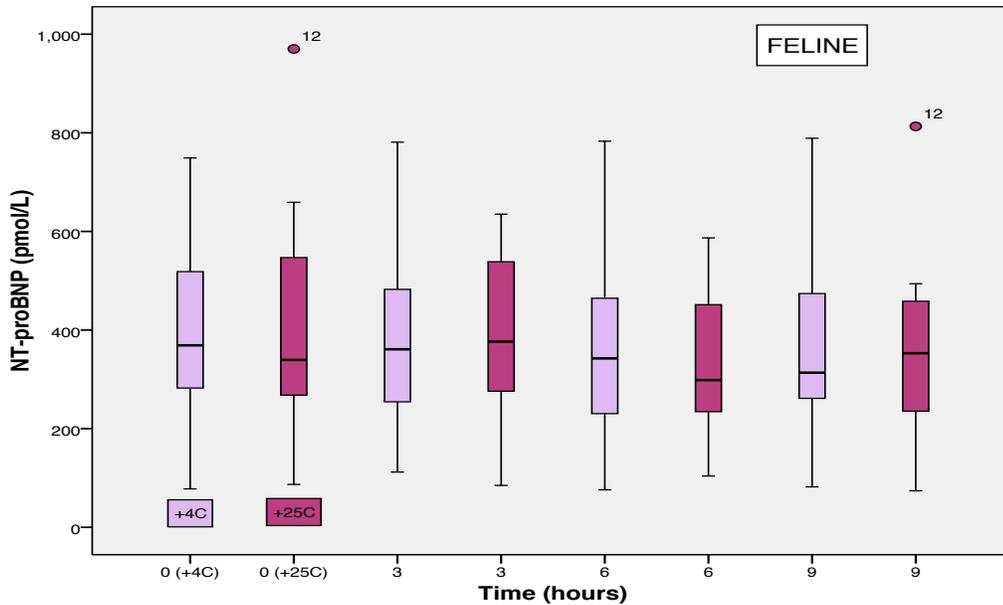


Study B

Temporal effect on feline and canine NT-proBNP concentration stored as whole blood in EDTA tubes at +4°C and +25°C for 9 hours

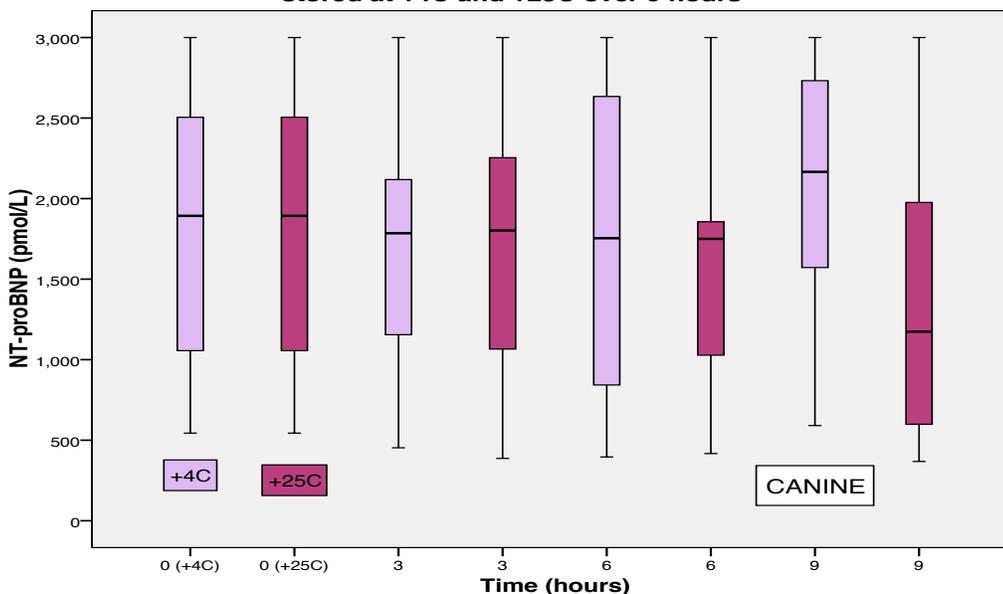
Feline NT-proBNP concentrations did not show statistically significant change (alpha 0.05) over 9 hours when stored at +4°C as whole blood in EDTA tubes ($p=0.053$) but did change significantly when stored at +25°C ($p=0.019$) (see Figure 19).

Figure 19 - Change in NT-proBNP concentration in FELINE unseparated blood stored at +4C and +25C over 9 hours



Canine NT-proBNP concentrations did not show statistically significant change (alpha 0.05) over 9 hours when stored at +4°C as whole blood in EDTA tubes ($p=0.132$) but did change significantly when stored at +25°C ($p=0.013$) (see Figure 20).

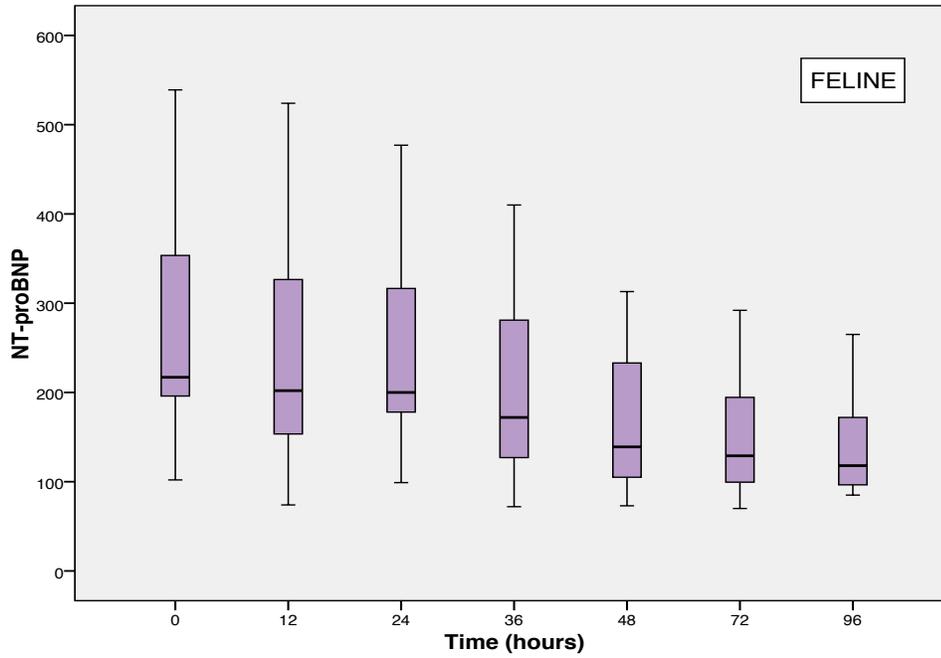
Figure 20 - Change in NT-proBNP concentration in CANINE unseparated blood stored at +4C and +25C over 9 hours



Study C

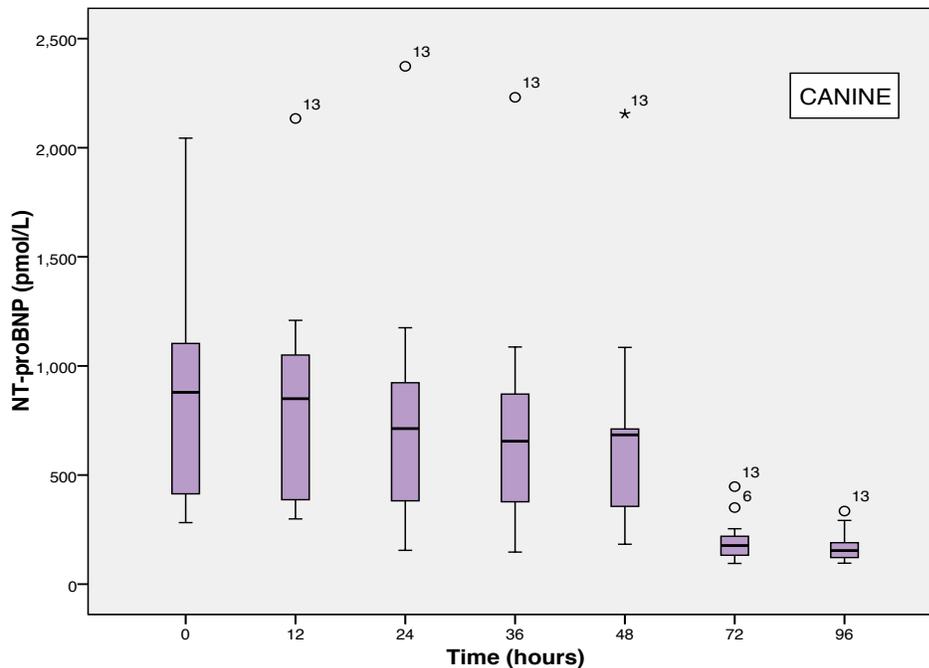
Temporal effect on feline and canine NT-proBNP concentration in frozen EDTA plasma when stored in ice packs at room temperature for 96 hours.

Figure 21 - Change in NT-proBNP concentration in FELINE PLASMA stored in ICE PACKS over 96 hours



Feline NT-proBNP in plasma stored in ice packs decreased over time ($p < 0.0001$). When compared to 0 hours, there was a significant difference at all time points (alpha 0.01), except at 12 hours ($p = 0.11$).

Figure 22 - Change in NT-proBNP concentration in CANINE PLASMA stored in ICE PACKS over 96 hours

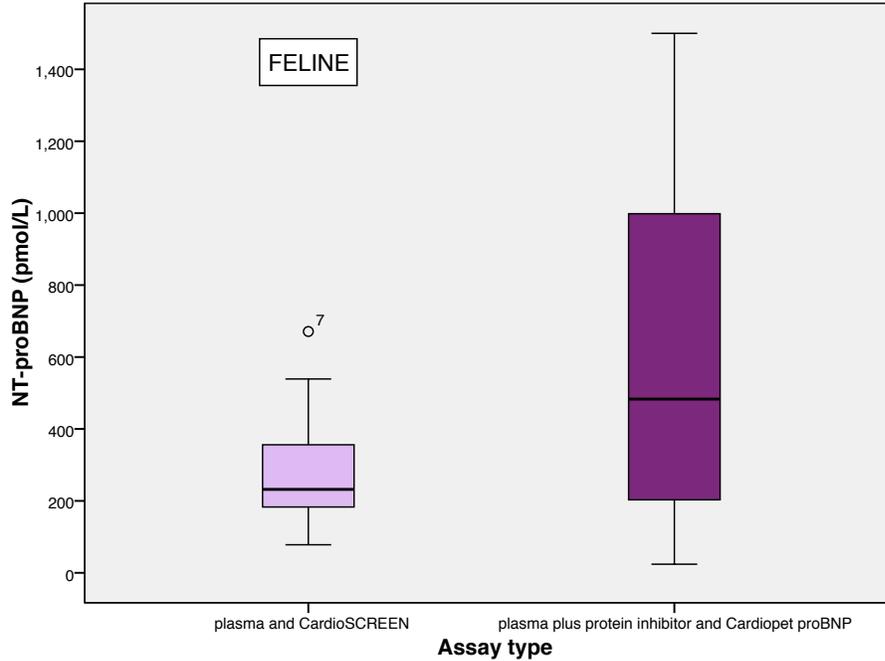


Canine NT-proBNP in plasma stored in ice packs decreased over time ($p < 0.0001$). When compared to 0 hours, there was a significant difference at all time points (alpha 0.01), except for 12 hours ($p = 0.1$) and 24 hours ($p = 0.047$).

Study D

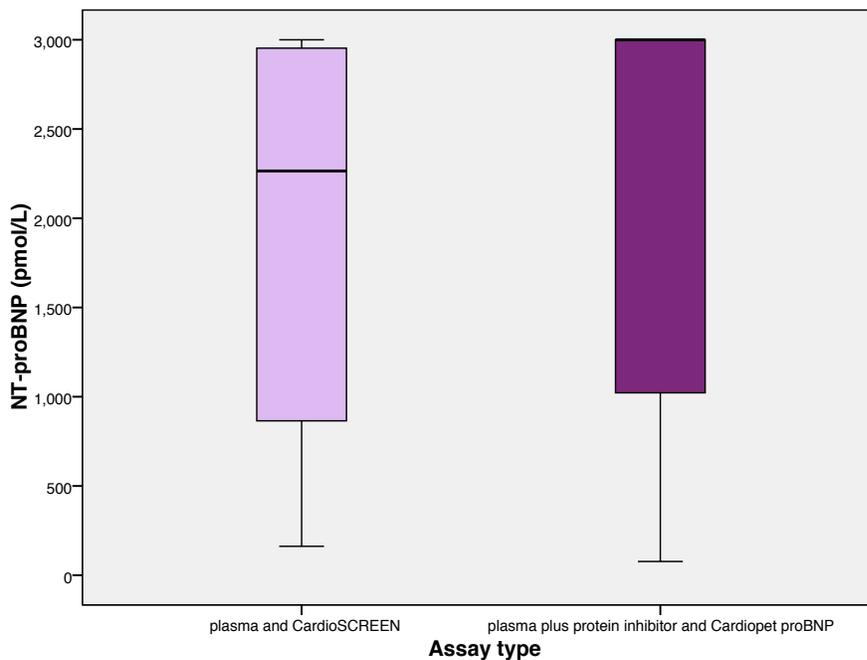
Comparison of NT-proBNP concentration in protease inhibited EDTA plasma measured using Cardiopet® proBNP versus NT-proBNP concentration in frozen EDTA plasma measured using CardioSCREEN

Figure 23 - Comparison of NT-proBNP concentration in FELINE PLASMA (measured using CardioSCREEN) versus PROTEIN-INHIBITED FELINE PLASMA (measured using Cardiopet proBNP)



Feline NT-proBNP in plasma without proteinase inhibitor (measured using CardioSCREEN) was lower than NT-proBNP in plasma with added proteinase inhibitor (measured using Cardiopet® proBNP) ($p < 0.0001$).

Figure 24 - Comparison of NT-proBNP concentration in CANINE PLASMA (measured using CardioSCREEN) versus PROTEIN-INHIBITED FELINE PLASMA (measured using Cardiopet proBNP)



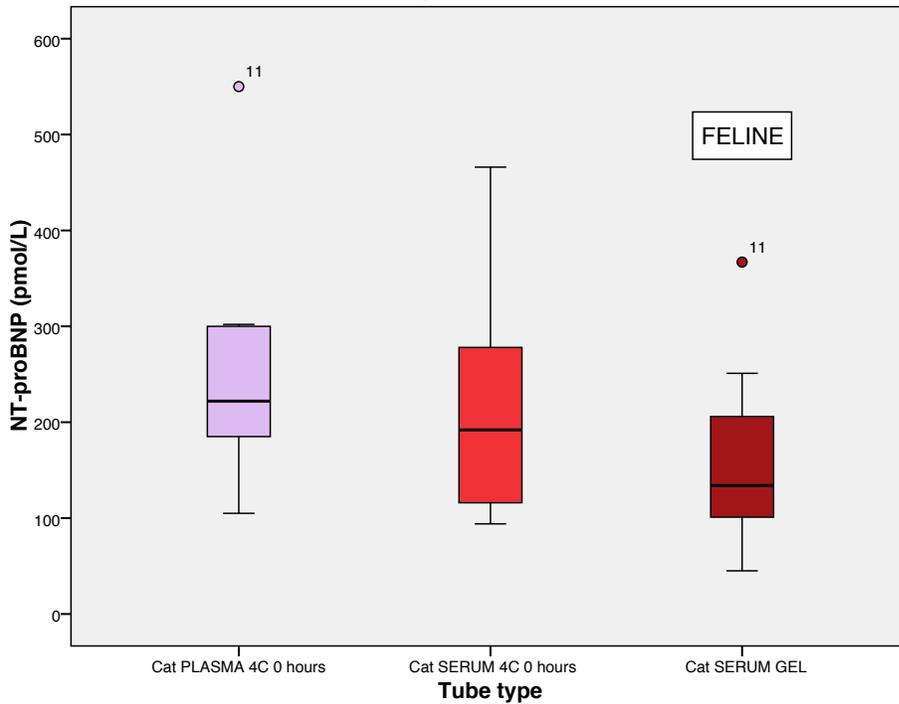
Canine NT-proBNP concentration in plasma without proteinase inhibitor (measured using CardioSCREEN) was lower than NT-proBNP concentration in plasma with added proteinase inhibitor (measured using Cardiopet® proBNP) ($p = 0.018$).

Median NT-proBNP concentration was >3000 pmol/L (off measureable scale) when using Cardiopet® proBNP.

Study E

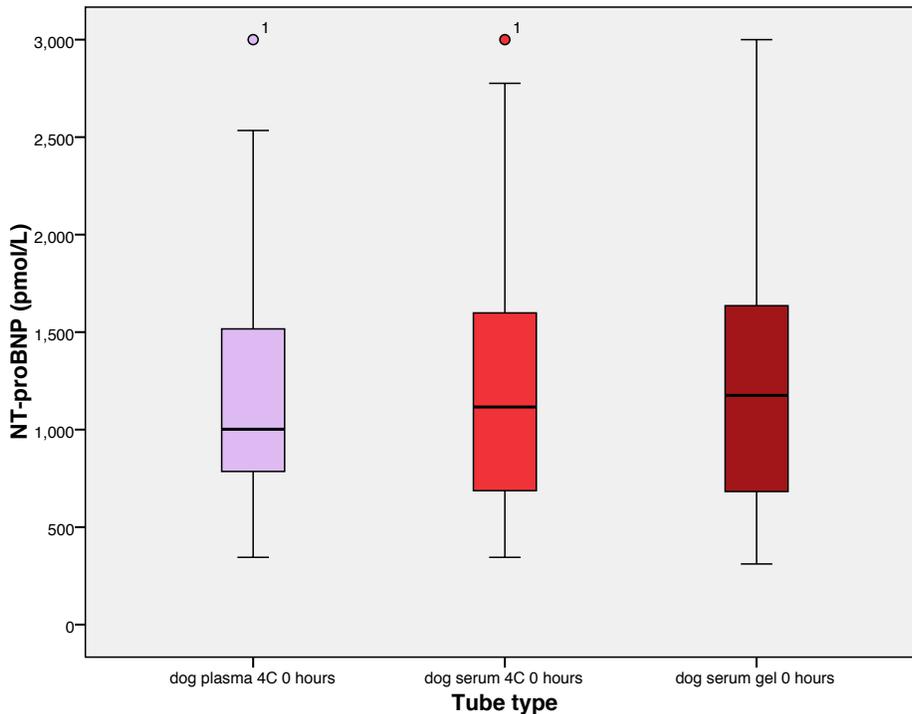
Effect of separator gel on feline and canine serum NT-proBNP concentration

Figure 25 - NT-proBNP concentration in FELINE PLASMA, SERUM and serum stored in separator GEL tubes



Feline NT-proBNP stored as serum in plain tubes was significantly different (alpha 0.05) when compared to concentration stored in serum gel tubes ($p=0.021$).

Figure 26 - NT-proBNP concentration in CANINE PLASMA, SERUM and serum stored in serum GEL tube

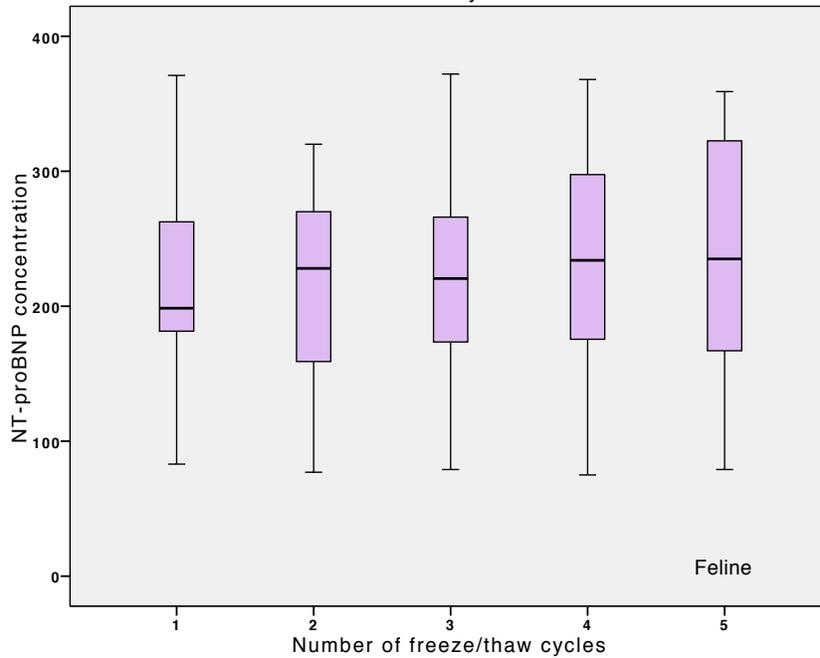


Canine NT-proBNP stored as serum in plain tubes was not significantly different (alpha 0.05) when compared to concentration stored in serum gel tubes ($p=0.11$).

Study F

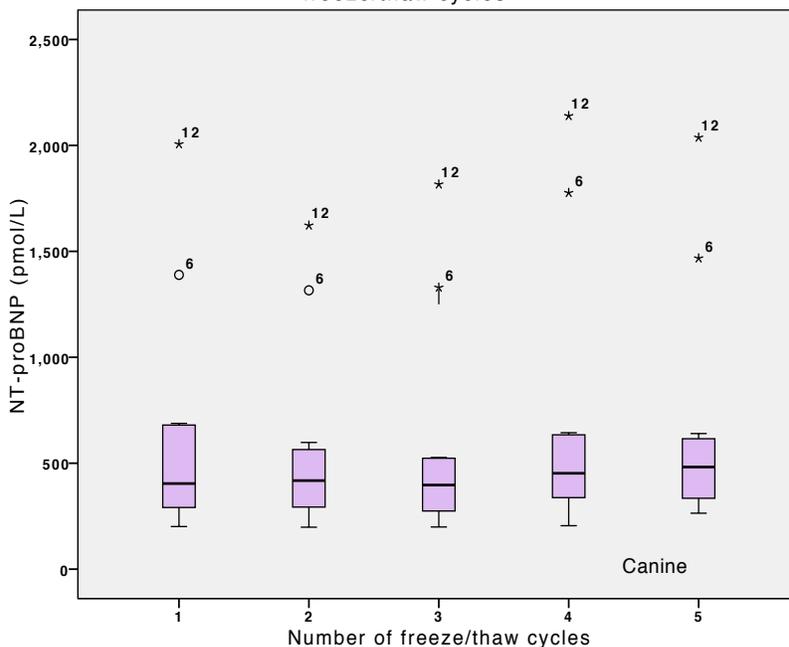
Effect of multiple freeze thaw cycles on feline and canine EDTA plasma NT-proBNP concentration

Figure 27 - NT-proBNP concentration in feline plasma after progressive freeze/thaw cycles



Feline plasma NT-proBNP did not show significant change over five freeze/thaw cycles ($p = 0.155$). When compared to a single freeze/thaw cycle ($\alpha 0.01$), there was no significant difference after two ($p = 0.695$), three ($p = 0.929$), four ($p = 0.239$) or five freeze/thaw cycles ($p = 0.308$).

Figure 28 - NT-proBNP concentration in canine plasma after progressive freeze/thaw cycles



Canine plasma NT-proBNP showed significant change over five freeze/thaw cycles ($p < 0.0001$). When compared to a single freeze/thaw cycle ($\alpha 0.01$), there was no significant difference after two ($p = 0.209$), three ($p = 0.06$), four ($p = 0.136$) or five freeze/thaw cycles ($p = 0.182$).

DISCUSSION

The aim of this study was to determine if sample preparation and shipping methods commonly employed in general veterinary practice might affect measured concentration of feline and canine NT-proBNP. The study has demonstrated that sample ageing, storage temperature and sample type all have significant effect on measured NT-proBNP concentration in cats and dogs, and must be considered when using NT-proBNP assay for clinical purposes.

Why does NT-proBNP bioreactivity change in blood samples?

Proteases are released when cells are disrupted and can quickly degrade protein, reducing the concentration of protein measured in a given sample. NT-proBNP bioreactivity decreases secondary to proteolytic degradation of the NT-proBNP molecule, therefore factors that increase (or decrease) proteolytic activity have an effect on measured concentration of NT-proBNP. Enzymatic activity is temperature dependent, increasing at higher temperatures and decreasing at lower temperature. It follows that proteolytic degradation of NT-proBNP will be greater at body temperature, and inhibited at sub-physiological temperatures. EDTA is a metalloproteinase inhibitor and may reduce proteolytic degradation of NT-proBNP, but may also cause NT-proBNP fragmentation. NT-proBNP bioreactivity in human EDTA plasma is reduced when compared to human heparinised plasma or serum (Ordonez-Llanos et al. 2008); however, this has not been observed in dogs. Historically NT-proBNP was measured in serum or EDTA plasma in cats and dogs. Later generations of feline and canine NT-proBNP assay specify that only EDTA plasma should be used for assay. Although not published, improved NT-proBNP stability in EDTA plasma compared to serum has been identified by the kit manufacturer (Beardow & Bilborough, 2010). Other sample-tube types have been shown to affect NT-proBNP and BNP measurements in humans, with changes noted in glass tubes versus plastic tubes (Lebrun et al., 2007) and in heparinized plasma versus serum (Di Serio et al., 2005). Separator gels are known to absorb some substances in blood such as oestradiol (Bush et al., 2001), but do not affect NT-proBNP concentration in human plasma (Dasgupta et al., 2003; Der Merwe et al., 2004).

Other factors may increase measured NT-proBNP concentration by altering epitope exposure to detector antibody. One study showed NT-proBNP increased by 18% in canine serum after freezing (Collins et al., 2010).

Is feline and canine NT-proBNP more labile than human NT-proBNP?

NT-proBNP in human serum and plasma shows good stability for up to 5 days when stored at room temperature (Lowbeer and Wallinder, 2007); whereas feline and canine NT-proBNP is relatively unstable (Connolly et al., 2011; Collins et al., 2010; Farace et al., 2008). One reason may be stability of the specific epitope detected in the feline and canine NT-proBNP assay. Interestingly one study compared

epitope stability in human NT-proBNP and showed epitopes located at N-terminal (1-12) degraded to 8-16% of initial immunological activity after 72 hours, whereas peptides at the 61-76 C-terminal proBNP degraded to 60-90%, and centrally located peptides (13-27) showed high stability (Katrukha *et al.*, 2005). Detection antibodies in feline and canine test kits are specific for peptides located at N-terminal (1-22), a similar position to the least stable peptides in human NT-proBNP.

Study findings compared to previous studies

Temporal effects

Significant serum and plasma NT-proBNP degradation occurred over time in feline and canine samples.

Temporal NT-proBNP degradation in feline serum has not been previously studied to the author's knowledge. NT-proBNP degradation in feline plasma stored at +25°C was similar in previous studies (see Table 3).

Table 3 - Percentage of baseline NT-proBNP in feline plasma after storage at +25°C

	24 hours	48 hours	72 hours
<i>This study</i>	67%	49.6%	35.8%
<i>Connolly et al., 2011</i>	62%	42%	35%
<i>Carrier et al., 2009</i>	32%	n/a	n/a

Median NT-proBNP in canine serum stored at +25°C decreased to 65% baseline concentration after 24 hours, similar to a previous study that showed 50% reduction (Collins *et al.*, 2010). Less degradation occurred in canine plasma with reduction to 92% baseline value by 24 hours when stored at +25°C, indicating EDTA may preserve NT-proBNP bioreactivity; however, another study showed degradation to 50% of baseline value in 2/3 of canine plasma samples by 24 hours (Farace *et al.*, 2008).

Storage temperature effects

This study showed that feline and canine NT-proBNP degrades less quickly in serum, plasma and whole blood stored at +4°C compared to +25°C, indicating that proteolytic degradation of NT-proBNP is inhibited but not stopped at lower temperatures. This is consistent with previous studies, which showed baseline NT-proBNP in feline plasma reduced to 88% after 24 hours when stored at +4°C, compared to 32% when stored at +25°C and 23% at +37°C (Carrier *et al.*, 2009). Another study showed canine plasma NT-proBNP did not decrease significantly after 24 hours when stored at +4°C, but degraded by up to 50% when stored at +25°C and +37°C (Farace *et al.*, 2008). There are no previous studies of NT-proBNP degradation in feline or canine whole blood to the author's knowledge; however, human NT-proBNP was stable in whole blood stored at room temperature at 24 hours (Barnes *et al.*, 2004).

NT-proBNP in frozen feline and canine plasma stored in ice packs at ambient temperature showed significant degradation over 96 hours. Temporal NT-proBNP degradation in feline and canine plasma stored on ice has not been previously studied to the author's knowledge. Median baseline feline NT-proBNP reduced to 93%, 79%, 64%, 59% and 54% at 24, 36, 48, 72 and 96 hours respectively. By comparison baseline NT-proBNP in feline plasma stored at +4°C (study C) had reduced to 72%, 61% and 48% at 24, 48 and 72 hours. Although ice packs delayed degradation, they failed to preserve NT-proBNP after 24 hours, with significant decrease noted by 36 hours.

Baseline canine NT-proBNP reduced to 96%, 86%, 80%, 79%, 22% and 19% at 12, 24, 36, 48, 72 and 96 hours respectively, similar to degradation noted in study A, indicating accurate measurements may not be achieved in canine samples shipped on ice (or stored at +4°C) later than 24 hours after sample collection.

Freeze-thaw cycle effects

There are no previous studies on the effect of multiple freeze/thaw cycles on feline or canine plasma to the author's knowledge; however, previous human studies showed no effect of multiple freeze/thaw cycles (Barnes et al., 2004; Mueller et al., 2004). Samples submitted for NT-proBNP assay are either analysed immediately, or more commonly frozen for analysis at a later date. Provided protocol is adhered to samples should not be subjected to more than one freeze/thaw cycle in clinical practice. Samples in study A and C were subjected to more than one freeze/thaw cycle, therefore the effect of multiple freeze/thaw cycles was tested. Median NT-proBNP in feline plasma did not change over five freeze/thaw cycles, although there was an upward trend. In dogs there was significant increase in measured NT-proBNP concentration over five freeze/thaw cycles; however, significant difference was not found with paired comparisons of single freeze/thaw cycle versus two, three, four of five freeze/thaw cycles (Wilcoxon signed rank, adjusted for multiple comparisons; alpha 0.01). NT-proBNP increased by 18% after the first freeze/thaw cycle in canine serum (Collins et al., 2010). A similar effect may occur in EDTA plasma and EDTA_{PI}, which was not tested in this study. Further studies are required to determine if NT-proBNP in EDTA_{PI} samples that have never been frozen might be lower than in samples that have been frozen for analysis at a later date.

Sample-tube effect

EDTA

NT-proBNP was higher in feline and canine EDTA plasma compared to serum at all time points including 0 hours. Another study showed similar findings with higher baseline NT-proBNP in feline plasma after storage at -20°C (254.5 pmol/L; IQR 75-471.5) compared to serum (151 pmol/L; IQR 32.5 – 390.5) (Gunther et al., 2010). When comparing NT-proBNP in canine serum versus plasma findings were

contrary to previous studies, which showed no significant difference (Kellihan et al., 2009; Boswood et al., 2008). Both studies used the VetSign™ Canine CardioSCREEN, manufactured by Guildhay, so comparison may be inappropriate.

One possible explanation for the difference in serum and plasma NT-proBNP observed in this study might be altered epitope expression caused by EDTA deformation of the NT-proBNP protein. However, it is more likely that NT-proBNP continued to degrade during the incubation process and that less degradation occurred in EDTA plasma samples.

Whole blood in EDTA tubes

NT-proBNP in feline and canine whole blood stored in EDTA tubes remained fairly stable over 9 hours when refrigerated (+4°C), suggesting that accurate NT-proBNP concentration can be obtained in unprocessed blood samples stored at +4°C for up to 9 hours. However, significant degradation was noted in feline and canine EDTA plasma at 12 hours when stored at +4°C, also difference in NT-proBNP in feline whole blood at 9 hours was close to statistical significance (p= 0.053), therefore practitioners should be encouraged to process samples as quickly as possible.

Seperator gel

NT-proBNP was significantly lower in serum stored in separator gel tubes than in plain tubes in cats, but not dogs. Although serum is no longer recommended for NT-proBNP assay, future assays may be developed to use serum without added proteinase inhibitor in order to improve clinical utility of the test. There have been no previous studies of separator gel effect in feline or canine NT-proBNP to the author’s knowledge. Two previous studies showed no difference in human serum versus serum stored in contact with SST gel at room temperature for 72 hours (Van Der Merwe et al., 2004; Dasgupta et al., 2003). Feline sample numbers were small and further studies may be required to confirm findings.

Cardiopet® proBNP proteinase inhibitor

NT-proBNP was significantly higher in feline and canine EDTA plasma_{PI} compared to EDTA plasma (see Table 4), similar to findings in two other recent studies (Gunther et al., 2010; McLane et al., 2011).

Table 4 – Feline NT-proBNP concentration in EDTA plasma with added proteinase inhibitor compared to EDTA plasma

<i>Study (number of cats)</i>	<i>NT-proBNP (pmol/L) in EDTA_{PI}</i>	<i>NT-proBNP (pmol/L) in plasma</i>
<i>This study (n = 36)</i>	486	232
<i>McLane et al., 2011 (n=18)</i>	794	646
<i>Gunther et al., 2010 (n = 20)</i>	278	254

Median NT-proBNP in canine EDTA plasma_{PI} was >3000 pmol/L, above the upper detection limit for the assay, and significantly higher than median NT-proBNP of 1916.5 pmol/L in EDTA plasma. Another study also showed that median NT-proBNP for 31 dogs with advanced mitral valve disease was outside measurable range, leading them to conclude that increasing the assay's upper limit of detection would improve the utility of the test (Reynolds et al., 2011).

Differences may be secondary to continued degradation in the EDTA plasma samples (without added proteinase inhibitor) during the analytical period. In this study EDTA plasma was processed and frozen within 60 minutes. Sample age including processing and incubation in the laboratory was not greater than 7 hours for feline samples (and 20 hours for canine samples), therefore the degree of change cannot be entirely attributed to temporal effect. Further studies are required to determine if incubation time has a significant effect on measured NT-proBNP, or whether proteinase inhibitor might actually cause differences.

Clinical significance of study findings

Algorithms are recommended for clinical interpretation of measured NT-proBNP, and patients are classified into one of three groups related to disease severity (see Figure 2_(a)). Current algorithm recommendations are more conservative when compared to historical algorithms (see Figure 2_(b)) and additional diagnostic investigation is now recommended when interpreting mid-range results (100-270 pmol/L for cats; 900 – 1800 pmol/L for dogs). However, there is a danger when using algorithms that the practitioner may over- or under-interpret quantitative results, particularly if close to cut-off values. In this study and previous studies, analysis of populations of NT-proBNP measurements showed an overall decrease in concentration over time, particularly at higher temperatures, which can affect test sensitivity and specificity. Furthermore, a significant number of individual samples showed increased NT-proBNP over repeated measurements, affecting the positive predictive value of the test. In order to determine how changes in NT-proBNP might affect clinical interpretation of *individual* results, animals in studies A, C and D were grouped according to algorithms (see Figure 2_(a)) using NT-proBNP measurements applicable to clinical practice (EDTA plasma stored at +4⁰C or on ice, or in EDTA_{PI}). Change in classification group was compared.

Cats - In Study A no cats had NT-proBNP < 100pmol/L, 13 cats had NT-proBNP between 100-270 pmol/L and 11 cats had NT-proBNP >270pmol/L. By 12 and 24 hours, 17% and 25% of samples respectively had sufficient degradation in NT-proBNP to cause reclassification (see Table 5).

Table 5 - Study A (24 cats) - Change in clinical interpretation group using algorithm for interpretation of NT-proBNP in plasma stored at +4°C

Time after sampling compared to 0 hours	12 Hours	24 Hours	48 Hours	72 Hours
Number (%) changed clinical classification group	4/24 (17%)	6/24 (25%)	6/24 (25%)	8/24 (33%)
Total number increased group	0	0	0	0
Total number decreased clinical group	↓ 4	↓ 6	↓ 6	↓ 8

In study C no cats had NT-proBNP<100 pmol/L, 10 cats had NT-proBNP between 100-270 pmol/L and 5 had NT-proBNP >270pmol/L. By 24 hours 33% of samples were sufficiently changed (2 increased and 3 decreased) to cause reclassification (see Table 6).

Table 6 - Study C (15 cats) - Change in clinical interpretation group using algorithm for interpretation of NT-proBNP concentration in plasma stored on ice

Time after sampling compared to 0 hours	12 Hours	24 Hours	36 Hours	48 Hours	72 Hours	96 Hours
Number (%) changed clinical classification	3/15 (20%)	5/15 (33%)	5/15 (33%)	7/15 (47%)	8/15 (53%)	10/15 (67%)
Total number increased group	↑ 2	↑ 2	↑ 2	0	0	0
Total number decreased group	↓ 1	↓ 3	↓ 3	↓ 7	↓ 8	↓ 10

In study D 11/36 (31%) cats had different clinical classification when comparing the two assay systems (see Table 7 and 8 below). When using CardioPet® more cats (8/11) were classified in the “significant cardiomyopathy likely” group; however, 3/11 cats increased one classification group (from normal to equivocal) in the CardioSCREEN group.

Table 7 - Study D (36 cats) Clinical classification using algorithm for interpretation of NT-proBNP concentration measured with VetSign™ CardioSCREEN versus CardioPet® proBNP

	<100 pmol/L	100-270 pmol/L	>270 pmol/L
VetSign™ CardioSCREEN	1	19	16
CardioPet®proBNP	4	8	24

Table 8 - Study D (36 cats) Difference in clinical classification groups using algorithm for interpretation of NT-proBNP concentration measured with VetSign™ CardioSCREEN versus CardioPet® proBNP

Number (%) changed clinical classification group	11/36 (31%)
Number CardioSCREEN increased one group compared to CardioPet®	3/11
Number CardioSCREEN decreased one group compared to CardioPet®	8/11

Dogs - In Study A 10/26 dogs had NT-proBNP <900 pmol/L, 13/26 dogs had NT-proBNP between 900-1800 pmol/L and 3/26 dogs had NT-proBNP >1800pmol/L. By 24 hours 19% of samples had sufficient change (2 increased and 3 decreased) in NT-proBNP to cause reclassification (see Table 9 below).

Table 9 - Study A (26 dogs) Change in clinical interpretation group using algorithm for interpretation of NT-proBNP concentration in plasma stored at +4°C

Time after sampling compared to 0 hours	12 Hours	24 Hours	48 Hours	72 Hours
Total number (%) changed clinical classification group	8/26 (31%)	5/26 (19%)	5/26 (19%)	4/26 (15%)
Total number increased one group	↑ 5	↑ 2	↑ 2	0
Total number decreased one group	↓ 3	↓ 3	↓ 3	↓ 4

In study C 9/15 dogs had NT-proBNP <900 pmol/L, 5/15 dogs had NT-proBNP between 900-1800 pmol/L and 1/15 dogs had NT-proBNP >1800pmol/L. By 24 hours 27% of samples had significant change (1 increased and 3 decreased) to cause reclassification (see Table 10 below).

Table 10 - Study C (15 dogs) - Change in clinical interpretation group using algorithm for interpretation of NT-proBNP concentration in plasma stored on ice

Time after sampling compared to 0 hours	12 Hours	24 Hours	36 Hours	48 Hours	72 Hours	96 Hours
Number (%) changed classification group	3/15 (20%)	4/15 (27%)	5/15 (33%)	7/15 (47%)	8/15 (53%)	10/15 (67%)
Total number increased group	↑ 1	↑ 1	↑ 1	0	0	0
Total number decreased group	↓ 1	↓ 3	↓ 3	↓ 4	↓ 6	↓ 6

In study D 17/65 (26%) dogs had different classification group when comparing the two assay systems (see Table 11). There were an equal number of dogs that increased or decreased one rank when comparing CardioPet® to CardioSCREEN. Three dogs were classified as probably normal using CardioSCREEN but were two ranks higher (heart failure probable) when using CardioPet®(see Table 12).

Table 11 - Study D (65 dogs) Difference in clinical classification group using algorithm for interpretation of NT-proBNP concentration measured with VetSign™ CardioSCREEN versus CardioPet® proBNP

	<900 pmol/L	900 - 1800 pmol/L	>1800 pmol/L
VetSign™ CardioSCREEN	19	12	34
CardioPet® proBNP	14	14	37

Table 12 - Study D (65 dogs) Difference in clinical classification groups using algorithm for interpretation of NT-proBNP concentration measured with VetSign™ CardioSCREEN versus CardioPet® proBNP

Number (%) changed clinical classification group	17/65(26%)
Number CardioSCREEN ↑ one group compared to CardioPet®	7/17 (41%)
Number CardioSCREEN ↓ one group compared to CardioPet®	7/17 (41%)
Number CardioSCREEN ↓ two group compared to CardioPet®	3/17 (18%)

Although statistical analysis of a population of NT-proBNP measurements (feline and canine plasma stored at +4°C, or on ice) showed significant change over time, the decrease in median values was relatively small between each time point. When using algorithms to clinically classify individual animals, there were very significant differences with both increased and decreased grouping. Approximately equal number of animals showed an increase or decrease in clinical group in samples less than 36 hours old; however, only decreasing clinical group was noted after 36 hours as samples degraded.

There was a marked difference in classification groups when comparing the two assay systems, with samples measured using the CardioPet® proBNP system much more likely to be in “heart disease/failure likely” group.

Study limitations

Test samples in studies A, B, C, E and F were not analysed in duplicate. The assay kit insert for VetSign CardioSCREEN™ stipulates that samples submitted for NT-proBNP assay are measured twice, and reported value is mean of the two results. The primary aim of the study was to assess the effect of variables on measurements, rather than assess the correlation of NT-proBNP to clinical findings; therefore, duplicate measurements were not considered necessary at each variable point. In fact most samples were subjected to multiple measurements and acted as their own controls. In total 1681 NT-proBNP measurements were obtained from 125 animals. Measuring all samples in duplicate would have allowed an assessment of intra-assay accuracy and would have been a useful addition to the study; however, funding was not available for such extensive testing.

Samples in studies A and C were subjected to two freeze/thaw cycles. Although it may have been possible to avoid two freeze/thaw cycles by aliquoting samples at the time of collection, it would have been difficult to achieve consistent methodology because samples were obtained during a normal working clinic. However, study F appears to show that there is no significant effect of up to 5 freeze/thaw cycles on feline and canine plasma.

Most test samples were stored for up to 30 days at -20°C, however some samples were stored for up to 6 months. NT-proBNP bioreactivity was within expected ranges in all samples tested; however, it is possible that some loss of bioreactivity may have occurred. Previous studies have shown apparently normal NT-proBNP bioreactivity in feline heparinized plasma when stored for up to ten years at -80°C (Lalor et al., 2009); however, one study showed slight but significant decrease of NT-proBNP concentration in human heparinized plasma stored at -20°C for two years (Cauliez et al., 2008).

All control samples were measured in duplicate and measured NT-proBNP concentrations were within “target” range. Co-efficient of variability (CV) for control samples was higher in this study compared to previous studies (see Table 13 and 14 below). CV was calculated using actual photometry readings (which are automatically recorded) rather than derived NT-proBNP concentration, which may have accounted for the differences noted in this study. Alternatively, differences observed may be related to change in assay kit performance, or may be operator dependent. A further analysis of variability in a control population of cats and dogs would have been a useful addition to this study and would have allowed an assessment of test accuracy.

Table 13 – Coefficient of variation for feline control samples

Control sample (Target measurement ± range) Estimated CV	Mean (± SD)	Low (230 ± 72 pmol/L) 31.3%	Medium (541 ± 180 pmol/L) 33%	High (1236 ± 524 pmol/L) 42%
This study (IDEXX CardioSCREEN)	17% (±2.7%)	18%	19%	14%
Wess et al., 2011 (Biomedica CardioSCREEN)	10.7%	14.3%	11.2%	6.7%

Table 14 – Coefficient of variation for canine control samples

Control sample (Target measurement ± range) Estimated CV	Mean (± SD)	Low (477 ± 131 pmol/L) 27%	Medium (1002 ± 202 pmol/L) 22%	High (1662 ± 312 pmol/L) 14%
This study (IDEXX CardioSCREEN)	15% (±5.9%)	20%	17%	9%
Boswood et al., 2008 (Biomedica CardioSCREEN)	7.1% (±1.1%)	n/a	n/a	n/a
Tarnow et al., 2009 (Biomedica CardioSCREEN)	15%	n/a	n/a	n/a

Assay kits must be stored at +2⁰C to +8⁰C. One canine CardioSCREEN assay kit was delayed for 48 hours during delivery to the laboratory; however, ice packaging was still frozen on arrival and the kit was presumed safe to use.

Potentially significant pre-analytical variables not studied included:

- Sampling site (cephalic versus jugular vein)
- Anxiety levels of the patient at sampling
- Lipid content of blood (visibly lipaemic samples were rejected)

Processing of samples was standardized as described in materials and methods. Processing variables not studied, which might have affected measured NT-proBNP, included:

- Centrifuge speed,
- Total centrifuge spin-time
- Centrifuge temperature

Time and temperature variables were controlled for studies A, B, E and F. Study C was designed to replicate transport conditions for sample sent to laboratories on ice, but findings may not accurately simulate typical transit conditions. Study C used only one method of ice-packaging. Some laboratories supply ice transport canisters, which may be more effective at maintaining EDTA plasma temperature below 0°C. Samples for study D were processed and shipped from the author's practice strictly according to current laboratory recommendations. It would have been useful to determine how quickly samples were processed upon arrival at IDEXX, also what percentage of sample were frozen for analysis at a later date versus analysed immediately; however, study D was designed to assess results obtained in field conditions.

When considering pre-analytical variables that might occur after dispatch from the veterinary practice, it would have been useful to have analysed variables such as;

- Time between arrival at the laboratory and re-freezing
- Comparison of samples that were analysed upon arrival at the laboratory versus samples re-frozen for analysis at a later date
- Comparison of samples that were processed within 24 hours of sample collection versus samples that were processed more than 24 hours after collection
- Comparison of samples stored at -20°C versus samples stored at -80°C

In addition to heart disease there are a number of potential physiological, pathological, pharmacological, biochemical and haematological factors shown to affect NT-proBNP levels in humans and animals which were not addressed in this study (Balion et al., 2008; Ordonez-Llanos et al., 2008; Kellihan et al., 2009; Lalor et al., 2009; Schmidt et al., 2009). Increased NT-proBNP concentration has been well documented in humans with renal failure, hypertension, diabetes mellitus, arrhythmia and pulmonary disease (Balion et al., 2008). Studies in people have demonstrated effects of age, sex, body weight and activity levels prior to sampling on measured NT-proBNP concentration (Ordonez-Llanos et al., 2008).

Diurnal variation was not assessed in this study. High day-to-day and weekly variability has been noted in previous studies of dogs and humans (Kellihan et al., 2009; Wu & Smith, 2004), but not in cats (Reynolds et al., 2010). An assessment of diurnal variability and concurrent intra-assay variability would have been useful.

The majority of animals in this study had heart disease. Animals that had concurrent disease such as renal failure, had non-cardiac disease or were normal were not excluded. It was presumed changes in measured concentrations were related to sample degradation (or proteinase inhibitor / tube effect) and that the *disease causing* elevated NT-proBNP concentration would not have an effect on subsequent sample degradation, which may not be true.

A normal control population of animals was not studied, as samples acted as their own controls. However, had normal animals been included in the study population, a sub-analysis of a normal versus diseased animals may have identified a difference in NT-proBNP degradation.

Conclusions

Obtaining accurate results

NT-proBNP bioreactivity is affected by a number of pre-analytical variables relevant to small animal practice including storage time, storage temperature and sample-tube type. Study results indicate that NT-proBNP concentration is relatively well preserved in EDTA plasma stored at +4⁰C (or on ice) for up to 24 hours, but degrades significantly after that time. Practitioners submitting plasma for NT-proBNP assay to laboratories using VetSign™ CardioSCREEN should process and freeze samples as quickly as is practical. Every effort should be made to ensure samples remain frozen in transit. Laboratories receiving frozen EDTA plasma should make every effort to store samples immediately upon arrival. Samples that have thawed in transit should be discarded unless the laboratory can document that samples were obtained less than 24 hours earlier. To this end practitioners submitting samples should be encouraged to note exact sampling time as well as sampling date on submission forms to avoid unnecessary disposal of usable samples.

NT-proBNP does not degrade significantly when stored as whole blood in EDTA tubes at +4⁰C for up to nine hours, therefore samples processed up to nine hours after the blood sample was taken should yield accurate NT-proBNP results, provided they have been stored in a refrigerator.

Interpreting results

Plasma NT-proBNP concentration measured using VetSign™ CardioSCREEN (with optimal sample processing and transit to the laboratory) was significantly lower than NT-proBNP concentration in EDTA plasma_{PI} measured using Cardiopet®proBNP. Current interpretation algorithms designed for use with Cardiopet®proBNP and VetSign™ CardioSCREEN are identical and IDEXX report good agreement

between the two assay systems (personal communication, Graham Bilborough, IDEXX). However, study findings indicate further validation is required, and that separate interpretative algorithms may be required for each of the two assay systems.

Median NT-proBNP concentration in canine EDTA plasma with added proteinase inhibitor measured using Cardiopet®proBNP was above the upper detection limit at >3000pmol/L. Although most of the dogs in this study had clinically significant heart disease, stratifying disease severity and deriving prognostic information using NT-proBNP measurements obtained with Cardiopet®proBNP was not possible. Although some studies have reported quantitative values >3000 pmol/L (up to 8407 pmol/L in a study by Serres et al., 2009), measurements outside the measureable range cannot be reliably determined using dilution methods (personal communication, Andy Beardow, IDEXX). Higher upper detection limits (and revised interpretative algorithms) will be required to fully realise the assay's ability to stratify disease severity and predict prognosis in dogs.

Historical recommendations for normal cut-offs are highly variable. Although the ability to differentiate heart disease from normal (or respiratory disease) is undisputed, actual recommendations for quantitative cut-off values are disparate. Changes in assay manufacturer, shifts in kit performance and incomplete understanding of sample handling effects have contributed to poor agreement. Recent studies, using quantitative NT-proBNP results with relatively high sensitivities and specificities have indicated potential utility for NT-proBNP as a prognostic indicator. Practitioners using NT-proBNP for this purpose should carefully study variables such as sample handling and assay system employed before using recommended cut-off values in individual studies to interpret NT-proBNP measurements.

Numerous studies have shown compelling data supporting a variety of clinical utilities for NT-proBNP in veterinary and human medicine, including disease screening. NT-proBNP may prove to be a useful and cost effective screening tool for heart disease in small animal practice, particularly in cats; however, wide coefficient of variability may limit the usefulness of single samples. Large population studies are required to determine test accuracy before widespread use could be recommended.

Practical limitations to use

NT-proBNP reliably discriminates dyspnea secondary to CHF in cats and dogs, making it a valuable test for the emergency assessment of cats and dogs presenting with breathing difficulty; however, results are rarely available within 24 hours and typically take 1-2 weeks to return because most laboratories run NT-proBNP assays in batches to limit costs (personal observation).

Future development

The commercial development of the feline and canine assay has been complex, and further redevelopments are likely to occur given the high operating costs of the current assay system. Although the CardioPet[®] proBNP and proteinase inhibitor tube system have reduced problems with NT-proBNP degradation in transit, its complexity has discouraged widespread use by general practitioners. Furthermore, the tube is expensive to manufacture, limiting its commercial viability (personal communication, Graham Bilborough, IDEXX). Change in assay design, perhaps using a more stable detector epitope, may negate the need for special sample handling and would improve the clinical utility of the test. Ultimately a reliable point-of-care assay would provide the most useful development for general practitioners. A point-of-care assay would negate most preanalytical variables and provide an instant NT-proBNP measurement, which would be a significant advance in the clinical use of NT-proBNP assay in small animal practice.

In the meantime practitioners must carefully follow laboratory protocol in order to minimize significant pre-analytical variability. When using NT-proBNP assay as a test for heart disease, practitioners must take care not to over- or under-interpret results, particularly values close to algorithm cut-offs. Intra-assay variability, diurnal variability, pre-analytical variability and variability between assay systems can all contribute to misleading results; therefore, NT-proBNP should not be used as an alternative to traditional diagnostic methods of diagnosing heart disease and failure. Nonetheless NT-proBNP assay does provide a useful ancillary tool for the management of heart disease in cats and dogs, provided it is used responsibly.

REFERENCES

1. Achen, S.E., Gordon, S.G., Roland, R.M., Boggess, M.M. & Miller, M.W. (2009) Serial evaluation of NT-proBNP in dogs with CHF predicts clinical score and the presence or absence of radiographic pulmonary edema [abstract]. *Journal of Veterinary Internal Medicine*; **23**: 687.
2. Adams, V.J., Evans, K.M, Sampson, J. & Wood, J.L.N. (2010) Methods and mortality results of a health survey of purebred dogs in the UK. *Journal of Small Animal Practice*; **51**(10): 512-524.
3. Balion, C.M., Santaguida, P., McKelvie, R., Hill, S.A., McQueen, M.J., Worster, A. & Raina, P.S. (2008) Physiological, pathological, pharmacological, biochemical and hematological factors affecting BNP and NT-proBNP. *Clinical Biochemistry*; **41**: 231-239.
4. Barnes SC, Collinson PO, Galasko G, Lahiri A, Senior R. (2004) Evaluation of N-terminal pro-B type natriuretic peptide analysis on the Elecsys 1010 and 2010 analysers. *Ann Clin Biochem*; **41**: 459-63.
5. Baumwart, R.D., Meurs, K.M. Assessment of plasma brain natriuretic peptide concentration in Boxers with arrhythmogenic right ventricular cardiomyopathy. *American Journal of Veterinary Research*; **66**: 2086 - 2089.
6. Beardow A.W. & Buchanan J.W. (2003) Chronic mitral valve disease in cavalier King Charles spaniels: 95 cases (1987-1991). *Journal of the American Veterinary Medical Association*; **203** (7): 1023–1029.
7. Beardow, A.W. & Bilborough, G. (2010) Introducing the New IDEXX Cardiopet® proBNP System [Letter]. IDEXX Reference Laboratory Wetherby, Grange House, Sandbeck Way, Wetherby, West Yorkshire.
8. Betti, I., Castelli, G., Barchielli, A., Beligni, C., Boscherini, V., De Luca, L., Messeri, G., Gheorghide, M., Maisel, A. & Zuppiroli, A. (2009) The role of N-terminal PRO-brain natriuretic peptide and echocardiography for screening asymptomatic left ventricular dysfunction in a population at high risk for heart failure. The PROBE-HF study. *Journal Cardiac Failure*; **15** (5): 377-84
9. Biondo, A.W., Ehrhart, E.J., Sisson, D.D., Bulmer, B.J., DeMorais, S.A. & Solter, P.F. (2003) Immunohistochemistry of atrial and brain natriuretic peptides in control cats and cats with hypertrophic cardiomyopathy. *Veterinary Pathology*; **40**(5): 501-506.
10. Bonnett, B.N., Egenvall, A., Hedhammar, Å. and Olson, P. (2005) Mortality in over 350,000 Insured Swedish dogs from 1995–2000: I. Breed-, Gender-, Age- and Cause-specific Rates. *Acta Veterinaria Scandinavica*; **46**: 105-120.
11. Boswood, A., Dukes-McEwan, J., Loureiro, J., James, R.A., Martin, M., Stafford-Johnson, M., Smith, P., Little, C. & Attree, S. (2008) The diagnostic accuracy of different natriuretic peptides in the investigation of canine cardiac disease. *Journal of Small Animal Practice*; **49**: 26-32.
12. Boswood, A. (2009) Biomarkers in cardiovascular disease: Beyond natriuretic peptides. *Journal of Veterinary Cardiology*; **11**, S23-S32
13. Borgarelli, M., Savarino, M., Crosara, S., Santilli, R. A., Chiavegato, D., Poggi, M., Bellino, C., La Rosa, G., Zanatta, R., Haggstrom, J. & Tarducci A. (2008) Survival Characteristics and Prognostic Variables of Dogs with Mitral Regurgitation Attributable to Myxomatous Valve Disease. *Journal of Veterinary Internal Medicine*; **22**:120–128.
14. Brenden CK, Hollander JE, Guss D, McCullough PA, Nowak R, Green G, Saltzberg M, Ellison SR, Bhalla MA, Bhalla V, Clopton P, Jesse R, Maisel AS. (2006) Gray zone BNP levels in heart failure patients in the emergency department: results from the Rapid Emergency Department Heart Failure Outpatient Trial (REDHOT) multicenter study. *American Heart Journal*; **151**:1006-11.

15. Buchanan, J.W. (1992) Causes and prevalence of Cardiovascular disease. In Kirk, R.W. and Bonagura, J.D. (eds): Kirk's Current Veterinary Therapy XI. Philadelphia, WB Saunders: 647.
16. Buchanan, J.W. (1999) Prevalence of Cardiovascular Disorders. In Fox, P.R., Sisson, DD., Moise, S.N. (editors): Textbook of Canine and Feline Cardiology: Principles and Clinical Practice, 2nd Edition, Philadelphia, WB Saunders: 464.
17. Bush, V.J., Janu, M.R., Bathur, F., Wells, A., Dasgupta, A. (2001) Comparison of BD Vacutainer SSTTM plus tubes with BD SSTTM II plus tubes for common analytes. Clin Chim Acta; **306**: 139-143
18. Carrier, A. Beardow A., Farace, G., Yeung, K., Ettinger, S.J. & Yee, K. (2009) Analytical Validation of a Commercially Available Feline N-Terminal Prohormone Brain Natriuretic Peptide ELISA [Abstract]. Journal of veterinary Internal Medicine; **23**:747.
19. Cauliez, B. Guignery, J, Marinier, S. Mariau, I & Lavoinne, A. (2008) Two-year stability of NT-proBNP in frozen samples using the Roche Elecsys system. Annals of Clinical Chemistry; **45**: 318-319.
20. Chetboul, V., Tessier-Vetzel, D., Escriou, C., Tissier, R., Carlos, C., Boussouf, M., Pouchelon, J.L, Blot, S. & Derumeaux, G. (2004) Diagnostic potential of natriuretic peptides in the occult phase of golden retriever muscular dystrophy cardiomyopathy. Journal of veterinary Internal Medicine; **18** (6): 845-850.
21. Chetboul, V., Serres, F. Tissier, R., Lefebvre, H.P., Sampedrano, C., Gouni, V., Poujol, L., Hawa, G. & Pouchelon, J.L. (2009) Association of plasma N-terminal pro-B-type natriuretic peptide concentration with mitral regurgitation severity and outcome in dogs with asymptomatic degenerative mitral valve disease. Journal of Veterinary Internal Medicine; **23** (5): 984-994.
22. Christ, M., Thuerlimann, A., Laule, K., Klima, T., Hochholzer, W., Perrouchoud, A.P. & Mueller, C. (2007) Long-term prognostic value of B-type natriuretic peptide in cardiac and non-cardiac causes of acute dyspnoea. European Journal of Clinical Investigation; **37**: 834-41.
23. Collins, S.A., Patteson, M.W., Connolly, D.J, Brodbelt, D.C., Torrance, A.G. & Harris, J.D. (2010) Effects of sample handling on serum N-terminal proB-type natriuretic peptide concentration in normal dogs and dogs with heart disease. Journal of Veterinary Cardiology; **12** (1): 41-8.
24. Connolly D.J., Soares Magalhaes R.J., Syme HM, Boswood A, Luis Fuentes V, Chu L. & Metcalf M. (2008) Circulating natriuretic peptides in cats with heart disease. Journal of Veterinary Internal Medicine; **22**: 96-105.
25. Connolly, D.J., Soares Magalhaes, R.J., Fuentes, V.L., Boswood, A., Cole, G., Boag, A. & Syme, H.M. (2009) Assessment of the diagnostic accuracy of circulating natriuretic peptide concentrations to distinguish between cats with cardiac and non-cardiac causes of respiratory distress. Journal of Veterinary Cardiology; **11**: S41-S50.
26. Connolly, D. (2010) Natriuretic Peptides: The Feline Experience. Veterinary Clinics of North America; **40** (4): 559-570.
27. Connolly, D.J, Hezzell, M.J, Luis Fuentes, V., Chang, Y.M., Swan, R., Syme, H.M. (2011) The effect of proteinase inhibition on the temporal stability of NT-proBNP in feline plasma at room temperature. Journal of Veterinary Cardiology; **13**: 13-19.
28. Darke, P. G. G. (1987) Valvular incompetence in cavalier King Charles spaniels. Veterinary Record; **120**: 365-366.
29. Di Serio F, Ruggieri V, Varraso L, De Sario R, Mastrotrilli A, Pansini N. (2005) Analytical evaluation of the Dade Behring Dimension RxL automated N-Terminal proBNP (NT- proBNP) method and comparison with the Roche Elecsys 2010. Clin Chem Lab Med; **43**: 1263-73.

30. Dasgupta, A. Chow, L., Tso, G. and Nazareno, L. (2003) Stability of NT-proBNP in Serum Specimens Collected in Becton Dickinson Vacutainer (SST) Tubes. *Clinical Chemistry*; **49**: 958-960.
31. DeFrancesco, T.C., Rush, J.E., Rozanski, E.A., Hansen, B.D., Keene, B.W., Moore, D.T. & Atkins, C.E. (2007) Prospective clinical evaluation of an ELISA B-type natriuretic peptide assay in the diagnosis of congestive heart failure in dogs presenting with cough or dyspnea. *Journal of Veterinary Internal Medicine*; **21**: 243-250.
32. Detweiler, D.K. & Patterson, D.F. (1965) The prevalence and types of cardiovascular disease in dogs. *Ann NY Acad Sci*; **127**: 481.
33. Ettinger, S., Beardow, A. and Yee, K. (2009) Cardiac biomarkers measured in 500 cats with heart disease, heart failure and other disease [Abstract]. In: Programs and abstracts of the 27th American College of Veterinary Internal Medicine Conference. Montreal, June 3-6.
34. Ettinger, S. (2010) NT-proBNP; The Cat is Not a Dog [Abstract]. In: Programs and abstracts of the 28th American College of Veterinary Internal Medicine Conference. Anaheim, June 9-12.
35. Farace G, Beardow A, Carpenter C, Yeung K, Zieba M, Ettinger SJ, Forney SD. (2008) Effect of shipping temperature on canine N-terminal prohormone atrial natriuretic peptide and N-terminal prohormone brain natriuretic peptide [Abstract]. *Journal of Veterinary Internal Medicine*; **22**:756.
36. Felker, M.G., Hasselblad, V., Hernandez, A.F & O'Connor, C.M. (2009) Biomarker-Guided Therapy In Chronic Heart Failure: A Meta-Analysis Of Randomized Controlled Trial. *American Heart Journal*; **158** (3): 422-430.
37. Fine, D.M., Declue, A.E. & Reiner, C.R. (2008) Evaluation of circulating amino terminal-pro-B-type natriuretic peptide concentration in dogs with respiratory distress attributable to congestive heart failure or primary pulmonary disease. *Journal of the American Veterinary Medical Association*; **232**:1674-1679.
38. Fioretti, M & Delli Carri, E. (1988) Epidemiological survey of dilatative cardiomyopathy in dogs. *Veterinaria*; **2**:81.
39. Fox, P.R., Oyama, M.A., MacDonald, K. & Reynolds, C.A. (2008) Assessment of NTproBNP Concentration in Asymptomatic Cats with Cardiomyopathy. *Journal of Veterinary Internal Medicine*; **22**: 719
40. Fox, P.R., Oyama, M.A., Reynolds, C., Rush, J.E., DeFrancesco, T.C., Keene, B.W., Atkins, C.E., Macdonald, K.A., Schober, K.E., Bonagura, J.D., Stepien, R.L., Kellihan, H.B., Nguyenba, T.P., Lehmkuhl, L.B., Lefbom, B.K., Moise, N.S. & Hogan DF. (2009) Utility of plasma N-terminal pro-brain natriuretic peptide (NT-proBNP) to distinguish between congestive heart failure and non-cardiac causes of acute dyspnea in cats. *Journal of Veterinary Cardiology*; **11**: S51-S61.
41. Gunther, C., Hildebrandt, N., Habedenteufel, E., Henrich, E., Schiller, S. & Schneider, M. (2010) Influence of Sample Preparation in Measurement of N-terminal Pro Brain Natriuretic Peptide (NT-proBNP) in Cats. [Abstract] In: Programs and abstracts of the 20th European College of Veterinary Internal Medicine Conference – Companion Animals, 9th - 11th September, Toulouse
42. Häggström, J., Hansson, K., Kvarn, C., Pedersen, H.D., Vuolteenaho, O., Olsson, K. (2000) Relationship between different natriuretic peptides and severity of naturally acquired mitral regurgitation in dogs with chronic myxomatous valve disease. *Journal of Veterinary Cardiology*; **2**: 7-16.
43. Häggström J., Hansson, K, Kvarn, C. & Swenson, L. (2002) Chronic valvular disease in the cavalier King Charles spaniel in Sweden. *Veterinary Record*; **131**(24): 549–553.
44. Häggström, J., Hoglund, K & Borgarelli, M. (2009) An update on treatment and prognostic indicators in canine myxomatous mitral valve disease. *Journal of Small Animal Practice*; **50**: S25-S33.

45. Hori, Y., Yamano, S., Iwanaga, T., Kano, T., Tanabe, M., Uechi, M., Kanai, R., Hoshi, F. & Higuchi, S. (2008) Evaluation of plasma C-terminal atrial natriuretic peptide in healthy cats and cats with heart disease. *Journal of Veterinary Internal Medicine*; **22**: 135-139.
46. Hsu, A., Kittleson, M.D. & Paling, A. (2009) Investigation into the use of plasma NT-proBNP concentration to screen for feline hypertrophic cardiomyopathy. *Journal of Veterinary Cardiology*; **11**: S63-S70.
47. Januzzi, J.L., Camargo, C.A., Saif Anwaruddin, S., Baggish, A.L., Chen, A.A., Krauser, D.G., Tung, R., Cameron, R., Nagurney, J.T., Chae, C.U., Lloyd-Jones, D.M., Brown, D.F., Foran- Melanson, S., Sluss, P.M., Lee-Lewandrowski, E. & Lewandrowski, K.B. (2005) The N-terminal Pro-BNP Investigation of Dyspnea in the Emergency department (PRIDE) study. *American Journal of Cardiology*; **95**: 948-54.
48. Januzzi, J.L, Chen-Tournoux, A.A. & Moe, G. (2008) Amino-terminal pro-B-type natriuretic peptide testing for the diagnosis or exclusion of heart failure in patients with acute symptoms. *American Journal of Cardiology*; **101**: 29-38.
49. Jourdain, P., Jondeau, G., Funck, F., Gueffet, P., Le Helleco, A., Donal, E., Aupetit, J.F., Aumont, M.C., Galliner, M., Eicher, J.C., Cohen-Solal, A. & Juliere, Y. (2007) Plasma brain natriuretic peptide-guided therapy to improve outcome in heart failure: the STARS- BNP Multicenter Study. *Journal of the American College of Cardiology*; **49**: 1733-1739.
50. Katrukha, A., Tolstaya, A., Antopolskii, M., Koshkina, E., Krasnoselskii, M., Kharitonov, M. & Seferian, M. (2005) The apparent stability of NT-proBNP significantly depends on the specificity of the antibodies utilized in the assay [Poster]. *American Association of Clinical Chemistry Annual Meeting*, July 24–28, Orlando, Florida.
51. Kellihan, H.B., Oyama, M.A., Reynolds, C.A. & Stepien (2009) Weekly variability of plasma and serum NT-proBNP measurements in normal dogs. *Journal of Veterinary Cardiology*; **11**, S93-97
52. Kirkwood, B.R. and Sterne, J.C. (2003) *Essential Medical Statistics*. Second Edition. Blackwell Science.
53. Lebrun, C, Neuder, Y., Pison, C., Chouri, N., barnoud, D., Belle, L. & Beaune, G. (2007) BNP or NT-proBNP: “that is the question” *Ann Biol Clin (Paris)*; **65** (5): 533-558.
54. Lowbeer, C. & Wallinder, H. (2007) Stability of IMMULITE 2000 plasma NT-proBNP in tubes with separator gel stored at room temperature (Letter to the Editor). *Clinica Chimica Acta*; **377**:283-284
55. Liu, S.K. (1977) Pathology of feline heart diseases. *Veterinary Clinics of North America*; **7**(2): 323- 39.
56. MacDonald, K.A., Kittleson, M.D., Munro, C. & Kass, P. (2003) Brain natriuretic peptide concentration in dogs with heart disease and congestive heart failure. *Journal of Veterinary Internal Medicine*; **17**: 172-177.
57. Maisel, A.S., Krishnaswamy, P., Nowak, R.M., McCord, J., Hollander, J.E., Duc, P., Omland, T., Sorrow, A.B., Abraham, W.T., Wu, A.H., Clopton, P., Steg, P.G., Westheim, A., Knudsen, C.W., Perez, A., Kazanegra, R., Herrmann, H.C. & McCullough, P.A. (2002) Rapid measurement of B- type natriuretic peptide in the emergency diagnosis of heart failure. *New England Journal of Medicine*; **347**: 161-167.
58. Maisel, A., Hollander, J.E., Guss, D., McCullough, P., Nowak, R., Green, G., Saltzberg, M., Ellison, S.R., Bhalla, M.A., Bhalla, V., Clopton, P. & Jesse, R. (2004) Primary results of the Rapid Emergency Department Heart Failure Outpatient Trial (REDHOT). A multicenter study of B-type natriuretic peptide levels, emergency department decision making, and outcomes in patients presenting with shortness of breath. *Journal of the American College of Cardiology* 2004; **44**: 1328-33.
59. Martin, M.W.S, Stafford Johnson, M. J., Celona, B. (2009) Canine dilated cardiomyopathy: a retrospective study of signalment, presentation and clinical findings in 369 cases. *Journal of Small Animal Practice*; **50**(1): 22-29.

60. Martin, M. W. S., Stafford Johnson, M. J. Strehlau, G. & King, J. N. (2010) Canine dilated cardiomyopathy: a retrospective study of prognostic findings in 367 clinical cases. *Journal of Small Animal Practice*; **51**(8): 428-436.
61. Masson, S., Latini, R., Anand, I.S., Vago, T., Angelici, L., Barlera, S., Missov, E.D., Clerico, A., Tognoni, G. & Cohn, J.N; Val-HeFT Investigators. (2006) Direct comparison of B-type natriuretic peptide (BNP) and amino-terminal proBNP in a large population of patients with chronic and symptomatic heart failure: the Valsartan Heart Failure (Val-HeFT) data. *Clinical Chemistry*; **52**: 1528-1538.
62. McLane, M., Baum, M., Allen, J., Farace, G., Ettinger, S., Beardow, A. (2011) Validation of Three Shipping Methodologies for NT-proBNP in a Mixed Cohort of Cats [Abstract]. *Journal of Veterinary Internal Medicine*; **25** (3): 656.
63. Moonarmart, W., Boswood, A., Luis Fuentes, V., Brodbelt, D., Souttar, K. & Elliott, J (2010) N-terminal pro B-type natriuretic peptide and left ventricular diameter independently predict mortality in dogs with mitral valve disease. *Journal of Small Animal Practice*; **51** (2): 84-96
64. Mueller, C., Scholer, A., Laule-Kilian, K., Martina, B., Schindler, C., Buser, P., Pfisterer, M. & Perruchoud, A.P. (2004) Use of B-type natriuretic peptide in the evaluation and management of acute dyspnea. *New England Journal of Medicine*; **350**: 647-54.
65. Mueller, T., Gegenhuber, A., Dieplinger, B., Poelz, W. & Haltmayer, M. (2004) Long-term stability of endogenous B-type natriuretic peptide (BNP) and amino terminal proBNP (NT- proBNP) in frozen plasma samples. *Clinical Chemistry Laboratory Medicine*; **42**: 942-944.
66. O'Donoghue, M., Chen, A., Baggish, A.L., Anwaruddin, S., Krauser, D.G., Tung, R. & Januzzi, J.L. (2005) The Effects of Ejection Fraction on N-Terminal ProBNP and BNP Levels in Patients With Acute CHF: Analysis From the ProBNP Investigation of Dyspnea in the Emergency Department (PRIDE) Study. *Journal of Cardiac Failure*; **11**: S9-S14.
67. Olsen, L.H., Fredholm, M. & Pedersen, H.D. (1999) Epidemiology and Inheritance of Mitral Valve Prolapse in Dachshunds. *Journal of Veterinary Internal Medicine*; **135**(5): 448–456.
68. Ordenez-Llanos, J., Collinson, P.O. & Christenson, R.H. (2008) Amino-terminal Pro-B-Type natriuretic peptide: Analytic Considerations. *American journal of Cardiology*; **101** (3): S9-15
69. Oyama, M.A., Sisson, D.D. & Solter, P.F. (2007) Prospective screening for occult cardiomyopathy in dogs by measurement of plasma atrial natriuretic peptide, B-type natriuretic peptide, and cardiac troponin-I concentrations. *American Journal of Veterinary Research*; **68**: 42-47.
70. Oyama, M.A., Fox, P.R., Rush, J.E., Rozanski, E.A. & Lesser, M. (2008) Clinical utility of serum N- terminal pro-B-type natriuretic peptide concentration for identifying cardiac disease in dogs and assessing disease severity. *Journal of the American Veterinary Medical Association*; **232**:1496-1503.
71. Oyama, M.A. Rush, J.E., Rozanski, E.A., Fox, P.R., Reynolds, C.A., Gordon, S.G., Bulmer, B.J., Lefbom, B.K., Brown, B.A., Lehmkuhl, L.B., Prosek, R., Lesser, M.B., Kraus, M.S., Bossbaly, M.J., Rapoport, G.S. & Boileau, J-S. (2009) Assessment of serum N-terminal pro-B-type natriuretic peptide concentration for differentiation of congestive heart failure from primary respiratory tract disease as the cause of respiratory signs in dogs. *Journal of the American Veterinary Medical Association*; **235** (11): 1319-1325.
72. Oyama, M & Singletary, E. (2010) The Use of NT-proBNP Assay in the Management of Canine Patients with Heart Disease. *Veterinary Clinics of North America*; **40**(4): 545-558.
73. Payne, A., Luis Fuentes, V., Boswood, A., Connolly, D., Koffas, H. & Brodbelt, D. (2010) Population characteristics and survival in 127 referred cats with hypertrophic cardiomyopathy (1997 to 2005). *Journal of Small Animal Practice*, **51**(10); 540–547.

74. Paige, C.F., Abbott, A., Elvinger, F. & Pyle, R.L. (2009) Prevalence of cardiomyopathy in apparently healthy cats. *Journal of the American Veterinary Medical Association*; **234**(11): 1398-1403.
75. Pfister, R., Tan, D., Thekkanal, J., Hellmich, M. & Schneider, C.A. (2007) NT-pro-BNP is associated with long-term outcome in a heterogeneous sample of cardiac inpatients. *European Journal of Internal Medicine*; **18**: 215-220.
76. Prosek, R., Sisson, D.D., Oyama, M.A. & Solter, P.F. (2007) Distinguishing cardiac and noncardiac dyspnea in 48 dogs using plasma atrial natriuretic factor, B-type natriuretic factor, endothelin, and cardiac troponin-I. *Journal of Veterinary Internal Medicine*; **21**: 238-242.
77. Reynolds, C.A., Kellihan, H.B., Alivernin, A., Singletary, G.E., Trafny, D.J., Stepien, R.L., Oyama, M.A. (2010) Weekly variability of plasma NT-proBNP measurements in cats with and without heart disease. [Poster] *Journal of Veterinary Internal Medicine*; **24** (3): 730.
78. Reynolds, C.A., Oyama, M.A., Rusk, J.E., Kellihan, H.B., Stepien, R.L., Lefbom, B.K., Weidman, J., Gordon, S.G., Lehmkuhl, L.B., Nguyenba, T.P., Meier, C.K., Fox, P.R. & Brown, D.C. (2011) NT-proBNP predicts first onset of congestive heart failure in dogs with degenerative mitral valve disease. [Abstract] *Journal of Veterinary Internal Medicine*; **25** (3): 650.
79. Richards, M. & Troughton, R.W. (2004) NT-proBNP in heart failure: therapy decisions and monitoring. *European Journal Heart Failure*; **6**: 351-354.
80. Riesen, S.C., Kovacevic, A., Lombard, C.W. & Amberger, C. (2007) Prevalence of heart disease in symptomatic cats: an overview from 1998 to 2005. *Schweiz Arch Tierheilkd*; **149**(2): 65-71.
81. Serres, F., Pouchelon, J.L., Poujol, L., Daste, T., Gouni, V. & Hawa, G. (2009) 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. *Journal of Veterinary Cardiology*; **11**(2): 103-121.
82. Singh, M.K, Cocchiario, M.F & Kittleson, M.D. (2010) NT-proBNP measurement fails to reliably identify subclinical hypertrophic cardiomyopathy in Maine Coon cats. *Journal of Feline Medicine and Surgery*; **12**: 942-947.
83. Singletary, G.E., Oyama, M., Rush, J.E., Fox, P.R., Stepien, R. L. (2011) NT-proBNP improves general practitioners accuracy and confidence of diagnosis in cats with respiratory signs. [Abstract] *Journal of Veterinary Internal Medicine*; **25** (3): 650.
84. Sisson, D.D. (2004) Neuroendocrine evaluation of cardiac disease. *Veterinary Clinics of North America*; **34**:1105-1126.
85. Sisson, D.D., Bulmer, B.J., Solter, P.F., Panico, R.R., Prosek, R.R., Oyama, M.A. & Hogan, D.F. (2008) Neurohormonal measurements in healthy dogs and dogs with acquired valvular or myocardial disease. [Abstract] In: Programs and abstracts of the 26th American College of Veterinary Internal Medicine Conference, San Antonio, Texas.
86. Smith, S. (2009) A prospective study of a population of 122 cats with naturally occurring cardiac or respiratory disease to evaluate the relevance of biomarkers and clinical signs (2006 - 2008) [Dissertation] Royal College of Veterinary Surgeons Diploma in Veterinary Cardiology Dissertation.
87. Tarnow, I., Olsen, L.H., Kwart, C., Hoglund, K., Moesgaard, S.G., Kamstrup, T.S., Pedersen, H.D., Häggström, J. (2009) Predictive value of natriuretic peptides in dogs with mitral valve disease. *Veterinary Journal*; **180** (2): 195-201.
88. Thomas, C.J. & Woods, R.L. (2003) Haemodynamic action of B-type natriuretic peptide substantially outlasts its plasma half-life in conscious dogs *Clinical and Experimental Pharmacology and Physiology*; **30**: 369-375.

89. Thrusfield, M. V., Aitken, C.G.G. and Darke, P.G.G. (1985) Observations on breed and sex in relation to canine heart valve incompetence. *Journal of Small Animal Practice*, **26**: 709-717.
90. Troughton, R.W., Frampton, C.M., Yandle, T.G., Espiner, E.A., Nicholls, M.G. & Richards, A.M. (2000) Treatment of heart failure guided by plasma amino terminal brain natriuretic peptide (N-BNP) levels improves outcome. *Lancet*; **355**: 1126-1130.
91. Troughton, R.W. & Richards, M.A. (2009) B-type natriuretic peptides and echocardiographic measures of cardiac structure and function. *Journal of the American College of Cardiology Cardiovascular Imaging*; **2** (2): 216-225.
92. Van Der Merwe, D., Henley, R., Lane, G., Field, R., Frenneaux, M., Dunstan, F. and McDowell, I. (2004) Effect of Different Sample Types and Stability After Blood Collection on N-terminal Pro-B-type Natriuretic Peptide as Measured with Roche Elecsys System. *Clinical Chemistry*; **50**(4): 779-780.
93. Valle, R., Aspromonte, N., Carbonieri, E., De Michele, G., Di Tano, G., Giovinazzo, P., Cioè, R., Di Giacomo, T., Milani, L., Noventa, F. & Chiatto, M. (2007) BNP-guided therapy optimizes the timing of discharge and the medium term risk stratification in patients admitted for congestive heart failure. *Monaldi Archive Chest Disease*; **68**:154-64.
94. Wagner, T., Luis Fuentes, V., Payne, J.R., McDermott, N, Brodbelt, D. (2010) Comparison of auscultatory and echocardiographic findings in healthy adult cats. *Journal of Veterinary Cardiology*; **12** (3): 171-182.
95. Wess, G., Daisenberger, P., Hirschberger, J. & Hartmann, K. (2008) The utility of NT-proBNP to differentiate cardiac and respiratory causes of dyspnea in cats [Abstract]. *Journal of Veterinary Internal Medicine*; **22**: 707-708.
96. Wess, G., Butz, V., Killich, M., Schulz, A., & Hartmann (2009) Evaluation of NT-proBNP in the Diagnosis of Various Stages of Dilated Cardiomyopathy in Doberman Pinschers [Abstract]. *Journal of Veterinary Internal Medicine*; **23** (3): 686.
97. Wess, G., Daisenberger, P., Hirschberger, J. & Hartmann, K. (2009) The utility of NT-proBNP to detect early stages of hypertrophic cardiomyopathy in cats and to differentiate disease stages. *Journal of Veterinary Internal Medicine*; **23**: 687
98. Wess, G., Schulze, A., Butz, V., Simak, J., Killich, M., Keller, L.J.M., Maeurer, J. and Hartman, K (2010) Prevalence of Dilated Cardiomyopathy in Doberman Pinschers in Various Age Groups. *Journal of Veterinary Internal Medicine*; **24**: 533–538.
99. Wess, G., Daisenberger, P., Mhling, M., Hirschberger, J. & Hartmann, K. (2011) Utility of measuring N terminal pro-brain natriuretic peptide in detecting hypertrophic cardiomyopathy and differentiating grades of severity in cats. *Journal of Veterinary Clinical Pathology*; **40** (2): 237-244.
100. Whitney, J.C. (1974) Observations of the effect of age on the severity of heart valve lesions in the dog. *Journal of Small Animal Practice*, **15** (8): 511-522.
101. Wu, A.H.B. & Smith, A. (2004) Biological variation of the natriuretic peptides and their role in monitoring patients with heart failure. *European Journal of Heart failure*; **6**: 355-358.
102. Zieba, M., Beardow, A., Carpenter, C., Farace, G., Yeung, K., Ettinger, S.J. & Forney, S.D. (2008) Analytical validation of a commercially available canine N-terminal prohormone brain natriuretic peptide ELISA [abstract]. *Journal of Veterinary Internal Medicine*; **22**: 756.

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APPENDICES

Table A – Utility of NT-proBNP in <u>cats</u> for identifying dyspnoea secondary to congestive heart failure (CHF)		
Author(s) (year)	Sample protocol and assay used	Comment
Ettinger (2010)	Venous blood into EDTA tubes, centrifuged at 4°C with 30 minutes. Placed in protease inhibitor tube and frozen at -80°C. Shipped to IDEXX, Westbrook on dry ice. Measured using IDEXX CardioPET system.	425 cats (727 samples) with heart disease ± CHF. Dyspnoeic cats with respiratory disease had median NT-proBNP of 82 pmol/L compared to 423 pmol/L in heart disease (not in failure) group and 873 pmol/L in CHF group.
Connolly et al. (2009)	1ml jugular blood collected into serum gel tubes, centrifuged within 20 minutes and stored at -20°C for 1-6 weeks, thereafter stored at -80°C. Measured using Guildhay Feline CardioSCREEN	74 cats with respiratory distress (41 non-cardiac, 33 CHF). Cats with CHF had higher median NT-proBNP at 523 pmol/L compared to respiratory group at 45 pmol/L). A cut-off value of 220 pmol/L discriminated CHF versus respiratory disease with sensitivity of 80.3% and specificity of 87.8%.
Fox et al (2009)	Blood collected into EDTA tubes, centrifuged within 1 hour, stored at -80°C for up to 4 weeks. Shipped on ice to IDEXX, Westbrook. Measured using IDEXX Feline CardioPET.	167 cats with dyspnoea (66 primary respiratory and 101 CHF). Median NT-proBNP was higher in CHF group at 754pmol/L (IQR 437-1035 pmol/L) compared to respiratory group at 76.5 pmol/L (IQR 24-180 pmol/L). A cut-off of 265 pmol/L discriminated CHF versus respiratory disease with a sensitivity of 90.2% and specificity of 85.3%.
Hsu et al (2009)	Blood collected into EDTA tubes, centrifuged within 1 hour and plasma stored at -20°C. Shipped on ice to VDI. Measured using CardioCARE.	40 Maine Coon cats, genotyped A31P heterozygous or negative, diagnosed with HCM by echocardiography. 9 cats were normal, 12 cats had equivocal HCM, 9 had moderate HCM and 10 had severe HCM. NT-proBNP cut-off value of 44pmol/L discriminated CHF from respiratory disease.
Wess et al. (2008)	Blood collected into EDTA tubes. Centrifuged at 4°C at 2000g for 10 minutes. Plasma placed in polypropylene (Eppendorf) tubes and stored for up to 6 months at -70°C. Measured using Biomedica Feline CardioSCREEN.	74 cats (33 healthy cats, 21 cats with respiratory disease and 20 cats with CHF). No significant difference between control and respiratory group, with mean NT-proBNP at 120 ± 107 pmol/L and 170 ± 143 pmol/L respectively. Mean NT-proBNP in cats with CHF was 686 ± 368 pmol/L. A cut-off value of 277 pmol/L discriminated CHF versus respiratory disease with a sensitivity of 95% and specificity of 84.6%.
Connolly et al (2008)	1ml of jugular blood collected into serum gel tubes, centrifuged within 20 minutes and stored at -20°C for 1-6 weeks, thereafter stored at -80°C. Measured using Guildhay Feline CardioSCREEN	<i>NT-proANP and NT-proBNP study.</i> 28 healthy cats, 17 cats with heart disease but not CHF and 33 cats with heart disease and CHF. Median (and 95% confidence intervals) NT-proBNP in control group was 33.6 pmol/L (11.2 - 56.1), median (and 95% confidence intervals) ANP was 682 pmol/L (95% CI 11.2-56.1). In heart disease but not failure group NT-proBNP was 184.1 pmol/L (111.0 - 257.1) and ANP was 1176.4 pmol/L (810.0 – 1542.9). In heart disease with CHF group NT-proBNP was 524.7 pmol/L (437.2 - 612.3) and ANP was 1865.0 (1499 – 2230.7). A cut-off value for NT-proBNP of 49 pmol/L discriminated normal cats from cats with heart disease with a sensitivity of 100% and specificity of 89.3%.

Table B – Utility of NT-proBNP and BNP in dogs to differentiate dyspnoea secondary to CHF from respiratory disease (or normal)

Authors (year)	Sample protocol and assay used	Comment
Oyama et al. (2009)	Venous blood collected into plain glass vacutainer, centrifuged within 1 hour, serum stored at -20°C. Shipped in cold packs. Measured by VDI using CardioCare	115 dogs with respiratory signs. Serum NT-proBNP cut-off concentration >1158 pmol/L discriminated between dogs with CHF and dogs with primary respiratory disease with a sensitivity of 85.5% and specificity of 81.3%
Tarnow et al. (2009)	Jugular blood collected into EDTA glass vacutainer, centrifuged at 3000g for 10 minutes at 5°C within 30 minutes. Plasma stored at -80°C. Measured using Guildhay CardioSCREEN	68 dogs comprising 13 healthy control dogs, 39 cavalier king Charles spaniels with pre-symptomatic mitral valve disease, 16 dogs with CHF. Cut-off values of 299 pmol/L were required to differentiate healthy dogs from dogs with moderate to severe mitral regurgitation with a sensitivity of 82% and specificity of 50%.
Boswood et al. (2008)	2 ml blood collected into EDTA tubes and plain tubes. Plasma and serum posted at ambient temperature to Guildhay for analysis, or frozen and posted as a batch for analysis at a later date.	77 dogs with respiratory or cardiac disease. A cut-off value of 210 pmol/L for EDTA plasma NT-proBNP differentiated dogs with heart disease from dogs with respiratory disease with a sensitivity of 85% and specificity of 82.4%.
Oyama et al. (2008)	Venous blood collected into plain glass vacutainer, centrifuged within 1 hour, serum stored at -20°C. Shipped in cold packs. Measured by VDI using CardioCare	119 dogs with MMVD, 18 dogs with DCM and 40 control dogs. Cut-off values of serum NT-proBNP > 445 pmol/L discriminated normal dogs from dogs with heart disease with a sensitivity of 83.2% and specificity of 90.0%.
Fine et al. (2008)	Venous blood collected into plain or EDTA glass vacutainers. Centrifuged at 1720g for 10 minutes with 20-30 minutes. Plasma or serum frozen at -20°C within 1 hour of collection. Submitted on dry ice to VDI. Measured using CardioCare	46 dogs with respiratory distress or cough. Dogs with CHF had significantly higher median serum or plasma NT-proBNP (1554 pmol/L; IQR 1651.5 - 3475.5 pmol/L) than dogs with primary pulmonary disease (357 pmol/L; IQR 192.5 - 565.5 pmol/L). NT-proBNP >1400 pmol/L had a sensitivity of 92% for detection of CHF.
Prosek et al. (2007)	Jugular blood collected into glass tubes containing EDTA and aprotinin. Centrifuged at 2500g for 15 minutes at 5°C, plasma stored at -70°C	48 dogs with dyspnea. BNP 12.18 pg/ml (95% CI 10.91-16.17 pg/ml) in respiratory group compared to 34.97 pg/ml (95% CI 23.51-52.02 pg/ml) in CHF group.
DeFrancesco et al. (2007)	1-7ml of venous blood collected in to EDTA tubes, centrifuged within 1 hour. Plasma stored at -80°C. Shipped on dry ice.	330 dogs, 75 normal, 76 with asymptomatic heart disease and 179 with cough or dyspnea signs. Dogs with CHF had higher median BNP (24.6 pg/ml) than dogs with non-cardiac signs (2.6pg/ml). Median BNP for ISACHC class I was 3.0pg/ml, class II 17.8 pg/ml and class II was 30.5pg/ml. BNP reliably differentiated cough or dyspnea caused by heart disease from respiratory disease. BNP was proportional to CHF severity.

Table C – Utility of natriuretic peptides for identification and determining severity of heart disease in cats		
Author	Sample protocol and assay used	Comment
Wess (2011)	Blood collected into EDTA tubes. Centrifuged at 4°C at 2000g for 10 minutes. Plasma placed in polypropylene (Eppendorf) tubes and stored for up to 6 months at -70°C. Measured using Feline CardioSCREEN, Biomedica.	<i>NT-proBNP study.</i> 201 cats; 99 normal and 102 with varying severity of HCM (9 equivocal, 15 mild, 17 moderate and 61 severe). Median NT-proBNP (IQR) pmol/L was; normal 18.9 (3.4-62.4), mild HCM 216 (87.6-392.5), moderate HCM 282.7 (131.9-466.6) and severe HCM 839.5 (655.3-1046.4). NT-proBNP concentration was significantly higher in all HCM groups compared to normal. No significant difference between mild vs. moderate, but significant difference between severe vs. mild/moderate group. Sensitivity and specificity for cut-off values of 49 pmol/L was 97.8% and 66%; >100 pmol/L was 92.4% and 93.9% and >150 pmol/L was 88% and 100%. Concluded that values of >100 pmol/L detected even mild HCM.
Fox et al (2011)	Blood collected into EDTA tubes. Centrifuged, separated, removed, frozen and stored at -20°C within 60 minutes of collection. Shipped on ice. Measured using either CardioPET by IDEXX, Westbrook or CardioSCREEN, Biomedica.	<i>NT-proBNP study.</i> 114 normal cats; 113 cats with occult cardiomyopathy. NT-proBNP reliably discriminated normal from OCM cats, with normal cut off 46pmol/L giving sensitivity of 85.8% and specificity 91.2%; cut-off >99 pmol/L giving sensitivity of 70.8% and specificity of 100%. Median NT-proBNP(IQR) (pmol/L) for normal cats was 24(24-32); cats with OCM 186 (79-479); HOCM 396 (205-686) and HCM 112 (48-318).
Ettinger (2010)	Venous blood into EDTA tubes, centrifuged at 4°C with 30 minutes. Placed in protease inhibitor tube and frozen at -80°C. Shipped to IDEXX, Westbrook on dry ice. Measured using CardioPET.	<i>NT-proBNP study.</i> 425 cats (727 samples) with heart disease ± CHF. NT-proBNP highly correlated with disease severity. Grading system 0 (normal), 1 (murmur, normal heart on echo), 2 (abnormal echo ± LA enlargement), 3 (abnormal echo, LA:Ao >1.7) ± clinical signs, 4 (heart failure). Median NT-proBNP (95% CI) for each group was; 0= 51 pmol/L (29-73); 1 = 153 pmol/L (95-212); 2 = 307 pmol/L (256-352); 3 = 610 pmol/L (480-702); 4 = 894 pmol/L (794 – 896). Recommended any cat with NT-proBNP > 100pmol/L has echocardiographic examination.
Hsu et al (2009)	Blood collected into EDTA tubes, centrifuged within 1 hour and plasma stored at -20°C. Shipped on ice to VDI. Measured using CardioCARE.	<i>NT-proBNP study.</i> 40 Maine Coon cats, genotyped A31P heterozygous or negative, diagnosed with HCM by echocardiography. 9 cats were normal, 12 cats had equivocal HCM, 9 had moderate HCM and 10 had severe HCM. Cats with severe HCM had higher NT-proBNP than all other groups at 134pmol/L (range 12-252).
Smith (2009)	Jugular blood collected into plain tubes. Centrifuged within 30 minutes. Serum was posted to laboratory to arrive within 24 hours, or frozen and shipped on ice at a later date. Some samples were analysed by Biomedica in Austria.	<i>NT-proBNP study.</i> 123 cats. Median (and IQR) NT-proBNP; 15 normal cats 1.3 pmol/L (0-16 pmol/L), 68 cats with cardiomyopathy 20.5 pmol/L (6-161 pmol/L), 13 cats with secondary cardiomyopathy 55 pmol/L (7.75-80.4 pmol/L), 8 cats with congenital HD 35.6 pmol/L (9.9- 167 pmol/L), 17 cats with respiratory 10.7 pmol/L (0-24.6 pmol/L). Median NT-proBNP (and IQR) for cats with heart disease grouped according to ISACHC. Class I (28 cats); 11.1 pmol/L (3.5-37.3), class IIa (17 cats); 19 pmol/L (2.3-54.2), Class IIb (17 cats); 11 pmol/L (0-301), class III (28 cats); 125.6 pmol/L (18.5 – 291).
Zimmering et al. (2009)	Jugular or cephalic blood sample into EDTA tube. Stored at room temperature and centrifuged within 5 hours. Stored at -20°C for up to 6 months. Analysed with proANP Biomedica.	<i>NT-proANP study.</i> 43 cats consisting 11 healthy cats, 16 cats with cardiomyopathy but not CHF and 16 cats with cardiomyopathy and CHF. Median NT-proANP concentration (range) differed significantly in each group at 381 pmol/L (52 – 450), 763 pmol/L (167-2386) and 2443 pmol/L (1189 – 15462) respectively.
Achen et al. (2009)	No information available	<i>NT-proBNP study.</i> 18 dogs with serial NT-proBNP measurement. NT-proBNP is significantly predictive of radiographic evidence of pulmonary oedema.
Connolly et al. (2008)	Blood collected into serum gel, stored for 20 minutes at room temperature then centrifuged. Serum stored at -20°C for 1-6 weeks then -80°C for up to 6 months. Measured using Vetsign Feline CardioSCREEN NT-proBNP, Guildhay, Surrey.	<i>NT-proANP and NT-proBNP study.</i> 28 healthy cats, 17 cats with heart disease but not CHF and 33 cats with heart disease and CHF. Median (95% CI) NT-proBNP in control group was 33.6 pmol/L (11.2 - 56.1), median (95% CI) ANP was 682 pmol/L (95% CI 11.2-56.1). In heart disease but not failure group NT-proBNP was 184.1 pmol/L (111.0 - 257.1) and ANP was 1176.4 pmol/L (810.0 – 1542.9). In heart disease with CHF group NT-proBNP was 524.7 pmol/L (437.2 - 612.3) and ANP was 1865.0 (1499 – 2230.7). A cut-off value for NT-proBNP of 49 pmol/L discriminated normal cats from cats with heart disease with a sensitivity of 100% and specificity of 89.3%.
Hori et al. (2008)	Blood into aprotinin tubes, centrifuged at 1500g 4°C for 10 minutes. Measured using human ANP assay	<i>C terminal-ANP study.</i> Experimental model of volume overloading. Ct-ANP concentration was strongly correlated with left atrial pressure. May provide additional information in diagnosis of heart disease in cats.
MacLean et al. (2006)	Blood collected into plastic EDTA tubes, centrifuged at 0°C and stored at -70°C.	<i>NT-proANP study.</i> 17 cats with HCM and 19 healthy cats. NT-proANP was higher in CHF cats (3808 fmol/L ± 1406) compared to healthy cats (3079 fmol/L ± 1233), but difference was not statistically significant (p = 0.11).

Table D - NT-proBNP and BNP for determining severity of heart disease in dogs		
Author	Sample protocol and assay used	Comment
Reynolds et al. (2011)	No information provided [abstract]	NT-proBNP measured in 66 dogs with MVD ISACHC-1b (LA:Ao>1.6). 31 developed radiographic pulmonary oedema, 35 remained asymptomatic. Median NT-proBNP of future CHF was 3001 pmol/L (IQR 2255-3001), versus 1600 pmol/L (IQR 984-2863) versus remaining asymptomatic. Sensitivity and specificity for NT-proBNP predicting if CHF would occur before next visit (2-9 months) was; NT-proBNP>1490 pmol/L, 90.3% and 48.6%; NT-proBNP >2150 pmol/L 77.4% and 68.6%.
Moonamart et al. (2010)	Jugular blood collected into EDTA or serum gel tubes. Stored at 4°C for up to 6 hours, then centrifuged at 1000g for 10 minutes at 40C. Plasma and serum stored at -80°C. Measured using CardioSCREEN, Guildhay.	73 dogs with mitral valve disease. NT-proBNP independent predictor of mortality in dogs with mitral valve disease. Increase in NT-proBNP by 100 pmol/L increased hazard of death by 7% (CI 2-11%)
Chetboul et al. (2009)	2mls of blood collected into EDTA, centrifuged at 40C within 1 hour, stored at -700C. Submitted to laboratory on dry ice. Measured using CardioSCREEN.	72 dogs with mitral valve disease and stage Ia and Ib ISACHC failure. NT-proBNP>466 pmol/L predicted decompensation (death or onset of CHF by 12 months) with sensitivity of 80% and specificity of 76%.
Serres et al. (2009)	2mls of blood collected into EDTA, centrifuged at 40C within 1 hour, stored at -700C. Submitted to laboratory on dry ice. Measured using CardioSCREEN.	74 dogs with mitral valve disease and stage II or III ISACHC heart failure. For all dogs NT-proBNP >1500 pmol/L predicted >6-month survival from non-survivor with a sensitivity of 80% and a specificity of 73%. Combining NT-proBNP and ISACHC classification, ISACHC stage II plus NT-proBNP<1265 pmol/L predicted 6-month survival and ISACHC stage III plus NT-proBNP > 2700 pmol/L predicted non-survival (median 5 days survival).
Tarnow et al. (2009)	Jugular blood collected into EDTA glass vacutainer, centrifuged at 3000g for 10 minutes at 50C within 30 minutes. Plasma stored at -80°C. Measured using Guildhay CardioSCREEN	53 dogs comprising 10 healthy control dogs, 27 cavalier king Charles spaniels with pre-symptomatic mitral valve disease (10 minimal MR, 10 moderate MR and 7 severe MR), 16 dogs with CHF. NT-proBNP concentration (IQR) in each group was 330 pmol/L (224-600); 276 pmol/L (237-318); 340 pmol/L (282-449); 626 pmol/L (580-705) and 1440 pmol/L (1042-1978) respectively. NT-proBNP correlated with severity of mitral regurgitation and heart failure group.
Achen et al. (2009)	No information provided [abstract]	18 dogs with serial NT-proBNP measurement. NT-proBNP is significantly predictive of clinical score and radiographic evidence of pulmonary oedema. A decrease in NT-proBNP ≥ 2000 pmol/L an improved clinical score was likely with 80% probability.
MacDonald et al. (2003)	Blood collected into polypropylene tubes containing EDTA and aprotinin. Centrifuged at 0°C and plasma stored at -70°C. Measured using canine BNP-32 radioimmunoassay.	34 dogs stratified into NYHA heart failure groups. BNP showed significant positive correlation with heart failure group. Weak correlation between BNP and left atrial diameter (r=0.43, p = 0.04). For every 10pg/ml increase in BNP mortality rate over 4 months increased by 44%.

Table E - NT-proBNP and BNP as screening tool for occult cardiomyopathy in dogs		
Author	Sampling, storage and assay used	Comment
Wess et al. (2011)	Blood collected from jugular vein into frozen EDTA tubes. Centrifuged at 2000g at +4°C for 10 minutes. Stored at -70°C. Measured using VetSign™ CardioSCREEN, Guildhay.	328 Doberman pinschers at various stages of pre-clinical DCM. NT-proBNP >400pmol/L detected all stages of DCM with sensitivity of 81.1% and specificity 75%. NT-proBNP >550 pmol/L predicted echocardiographic changes with sensitivity of 90% and specificity of 75%.
Oyama et al. (2009)	No information available [abstract]	100 Doberman pischers prospectively screened for DCM with Holter, echo and NT-proBNP. 33 had DCM (5 echo positive, 16 Holter positive, 12 echo and Holter positive. NT-proBNP of >478 pmol/L showed 88.2% sensitivity and 91.4% specificity for detecting echo-positive dogs. Using Holter plus NT-proBNP >478pmol/L had 86.4% sensitivity and 96.8% specificity (93% accuracy) for detecting occult cases.
Oyama et al. (2007)	Blood collected into chilled plastic tubes containing EDTA and aprotinin. Centrifuged at +5°C at 1100g for 15 minutes. Transferred to cryotubes and stored at -80°C. C-terminal BNP measured using RK-011-22 by Phoenix Pharmaceuticals.	118 dogs. Concentration of >6.21 ng/ml BNP had sensitivity of 95.2% and specificity of 61.9% for identifying occult DCM. cTn-I and ANP had relatively low predictive values.
Baumwart et al. (2005)	Jugular blood sample into polypropylene tubes containing EDTA and aprotinin at 0°C. Centrifuged at 1600g at 0°C for 15 minutes. Stored at -80°C. Measured using canine-32 BNP, Penninsula Laboratories.	Mean±SD BNP concentration for ARVC Boxers was not significantly different from normal Boxers.
Chetboul et al. (2004)	Blood collected into chilled plastic tubes containing proteinase inhibitors (aprotinin, trypsin inhibitor and phenylmethyl-sulfonyl fluoride), centrifuged at +4°C and 3000g, stored at -60°C.	50 male Golden retrievers, 29 healthy controls and 21 with X-linked muscular dystrophy. In dogs >1 year old BNP >65 pg/ml identified GRMD with sensitivity of 78% and specificity of 86%.

Table F – NT-proBNP cut-off values, sensitivity and specificities for differentiating CHF as cause of dyspnea in dogs

Author	Sampling, storage and assay used	NT-proBNP cut-off	Population studied	Sensitivity	Specificity
Oyama et al. (2009)	Centrifuged within 60 minutes of collection, serum stored at -20°C, shipped in cold packs to laboratory. Analysed by Veterinary Diagnostics Institute.	>1158 pmol/L	Dogs with CHF versus dogs with primary respiratory disease	85.5%	81.3%
Tarnow et al. (2009)	Centrifuged within 30 minutes of collection at +5°C and 3000g, EDTA plasma stored at -80°C. Analysed using Guildhay VetSign Canine CardioScreen.	>299 pmol/L	Healthy dogs versus CKCS with <u>moderate to severe</u> mitral regurgitation	82%	50%
		454 pmol/L	Healthy dogs versus CKCS with <u>severe mitral</u> regurgitation	100%	80%
		>299 pmol/L	Healthy dogs versus CKCS with <u>severe mitral</u> regurgitation	100%	43%
Boswood et al. (2008)	Serum and EDTA plasma frozen at -80 °C for analysis at a later date, or posted directly to the laboratory. Analysed using Guildhay VetSign Canine CardioScreen.	>210 pmol/L	dogs with heart disease from dogs with respiratory disease	85%	82.4%
Oyama et al. (2008)	Centrifuged within 60 minutes of collection, stored at -20°C, shipped in cold packs (+4°C) to laboratory. Analysed by Veterinary Diagnostics Institute.	> 445 pmol/L	Normal dogs from dogs with heart disease (MMVD and DCM)	83.2%	90.0%
Fine et al. (2008)	Blood centrifuged at 1720g for 10 minutes within 60 minutes of collection. Serum and EDTA plasma stored at -20°C. Analysed using Guildhay VetSign Canine CardioScreen.	>1400 pmol/L	Dogs with CHF versus primary pulmonary disease	92%	n/a