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Detection by ELISA of C-terminal proBNP in plasma from cats with cardiomyopathy

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ABSTRACT

The B-type natriuretic peptide prohormone (proBNP) is enzymatically cleaved into an inactive N-terminal peptide and a biologically active C-terminal peptide with many beneficial cardiorenal effects. The purpose of this study was to develop and test in cats with cardiomyopathy an immunoassay to quantify the concentrations of C-terminal proBNP in feline plasma.

An anti-canine proBNP monoclonal antibody (UI-1021) was shown to have adequate binding affinity to proBNP 80–106 for use in a solid-phase immunoassay, and by epitope mapping to bind within positions 84–87 of feline proBNP. UI-1021 was paired with an affinity-purified rabbit polyclonal detection antibody to feline proBNP 100–106, in a sandwich ELISA with feline proBNP 80–106 standard. The linearity and analytical range and sensitivity of the assay were confirmed from 1.4 to 85 pmol/L. Spike recovery averaged 106.5% (95% confidence interval 78–135%). Within run and intra-assay coefficients of variation were <12%. A protease inhibitor mixture preserved proBNP 80–106 immunoreactivity for at least 5 days in plasma. Clinical verification of the ELISA was done using plasma from 13 cats with cardiomyopathy, whose C-terminal proBNP concentrations ranged from 1.7 to 78.8 pmol/L vs. <1.4–1.8 pmol/L in plasma from 18 healthy cats.

Concentrations were found to be substantially lower than reported N-terminal proBNP concentrations, and similar to those of human heart failure patients where relative C-terminal BNP deficiencies have been proposed as contributory to the progression of the disease.

Introduction

The B-type natriuretic peptide prohormone (proBNP), produced by myocardiocytes, is enzymatically cleaved into biologically active C-terminal and inactive N-terminal peptides. By binding to specific receptors on sensitive cells, C-terminal peptide (BNP) induces natriuresis and diuresis, and other cardiorenal actions that counteract the progression of congestive heart failure (CHF) (Nishikimi et al., 2006; Woodard and Rosado, 2008; Potter et al., 2009).

Clinical assays for human N-terminal, C-terminal, and uncleaved proBNP have been developed, and their relative diagnostic and prognostic utility shown (Prontera et al., 2004; Masson et al., 2006; McKie et al., 2006; Waldo et al., 2008; Miller et al., 2009; Macheret et al., 2011; Jensen et al., 2012; Costello-Boerrigter et al., 2013; Geske et al., 2013). They have also helped explain a clinical paradox of the proBNP system, namely, that despite high circulating concentrations of immunoreactive proBNP isoforms, CHF patients retain fluid and sodium but respond to exogenously administered BNP (Goetze et al., 2003; Menon et al., 2009). The paradox is postulated to reflect an endocrine disorder caused not only by natriuretic peptide resistance but importantly also by a relative deficiency of biologically active BNP adequate to meet physiological needs (Chen, 2007; Lam et al., 2007; Liang et al., 2007; Dries, 2011; Nishikimi et al., 2011; Baerts et al., 2012; McKie and Burnett, 2015).

Comparatively little is known about the feline proBNP system. Feline proBNP comprises 106 amino acids (Liu et al., 2002), most likely with a 17-amino acid ring formed by a cys–cys disulfide bond near its C-terminus (Fig. 1). The site of cleavage of feline proBNP has not been confirmed, but is likely after proBNP 79, thereby forming a biologically active 26-amino acid peptide (proBNP 80–106), or longer. Feline proBNP immunoreactivity is found within myocardiocytes of the atria and ventricles, and ventricular expression increases with cardiomyopathy (Biondo et al., 2003).

A recently developed sandwich ELISA to measure feline N-terminal proBNP has been shown to have diagnostic value (Connolly et al., 2008, 2009; Fox et al., 2009; Lalor et al., 2009), and

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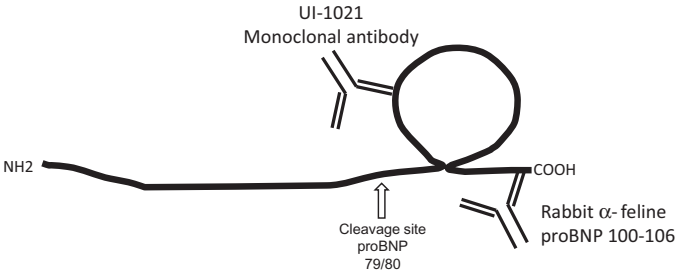

Fig. 1. Structure of feline proBNP and sites of antigenic determinants used in this study. Myocardiocytes are believed to produce the prohormone, which is enzymatically cleaved to a physiologically active C-terminal peptide, containing a 17-amino acid ring, and a nonfunctional N-terminal fragment. The putative site of cleavage between amino acid residues 79 and 80 is shown by the arrow, along with the relative binding sites of the two antibodies used in this study, UI-1021 and anti-feline proBNP 100–106.

a competitive radioimmunoassay to measure canine C-terminal proBNP was successfully used to find C-terminal proBNP in the plasma of cats with cardiomyopathy (Sisson et al., 2003). Comparison of the absolute amounts of feline C-terminal and N-terminal proBNP from these studies suggests that most of the circulating proBNP immunoreactivity in cats with cardiomyopathy represents the biologically inactive N-terminal peptide. Hence, feline patients with cardiomyopathy seem to demonstrate elements of the BNP paradox.

The purpose of the current study was to develop a feline-specific immunoassay that could be used to confirm the circulating concentrations of plasma C-terminal proBNP in cats with cardiomyopathy.

Materials and methods

Production and preparation of test reagents

An affinity purified rabbit polyclonal antibody made to the 7-amino acid C-terminus of feline proBNP (proBNP 100–106) was prepared for use in the ELISA. The ELISA test standard is a synthetic cyclized feline proBNP 80–106.

The ELISA capture antibody is an affinity purified mouse monoclonal IgG_1K antibody (UI-1021) developed to a 12-amino acid sequence of canine proBNP (Solter et al., 2008). The binding affinity of UI-1021 was determined by estimation of its affinity constant (K_{aff}) to proBNP 80–106, using a solid-phase ELISA technique (Beatty et al., 1987). Briefly, serial dilutions of feline proBNP 80–106 were added to the wells of a clear, flat bottom 96-well microtiter plate (Nunc Maxisorp) coated with the anti-proBNP 100–106 antibody. The wells were subsequently treated with UI-1021, followed by alkaline phosphatase-conjugated rabbit anti-mouse antibody (Sigma-Aldrich), and then a fluorescent substrate (Attophos). The relative fluorescence units (RFUs) of each well were measured with a fluorometer (SPECTRA-max GEMINI XS). The concentrations of UI-1021 vs. RFUs for each of the proBNP 80–106 concentrations were plotted and used to calculate the affinity constant (K_{aff}). The mean and 95% confidence interval of K_{aff} were calculated from the values obtained at the various concentrations.

Linear mapping of the binding epitope of UI-1021, and cross-reactivity to other natriuretic peptides, was assessed using the C-terminal 32 amino acids of human, rat, and dog proBNP, as well as C-terminal human atrial natriuretic peptide (ANP 5–28) and C-type natriuretic peptide (CNP 22). Based on the GenBank online database and published reports (Biondo et al., 2002), the amino acid sequence of feline ANP 5–28 and CNP 22 is likely identical to the human peptides (Table 1). All of the peptides were obtained from Phoenix Pharmaceuticals.

Two ELISA protocols were used for the linear mapping experiments. The first protocol consisted of adsorbing serial dilutions of each of the natriuretic peptides directly onto wells of a clear microtiter plate, followed by UI-1021. Alkaline phosphatase conjugated goat anti-mouse antibody (Sigma-Aldrich) and the fluorescent substrate (Attophos) were then applied to each well after washing, and the RFUs of each well measured. The experiment was repeated as a competitive binding assay by coating microtiter plate wells with UI-1021, followed by a saturating amount of each peptide.

Feline C-terminal proBNP ELISA description and validation

The sample buffer contains 0.05% Tween-20 (Sigma-Aldrich), 0.1% sodium azide (Sigma-Aldrich), StartingBlock (Pierce), and a mixture of the protease inhibitors (PI), D-Phe-Phe-Arg-chloromethylketone (PPACK II, Calbiochem), and benzamidine and aprotinin from bovine lung (Sigma-Aldrich). Feline EDTA plasma samples are diluted before assay by 50% in Sample buffer. For preparation of test calibration curves, canine EDTA plasma is diluted 50% in the sample buffer and spiked with feline proBNP 80–106. The ELISA plate wash buffer is 0.05% Tween-20, 0.1% sodium Azide in Tris buffered saline (Sigma-Aldrich).

Both test standards and samples are prepared for ELISA by first removing potentially interfering antibodies through incubating each standard and diluted sample with Protein A conjugated agarose beads (Protein A Plus, Pierce Biotechnology) in microspin filter columns with 0.45 μm cellulose acetate membranes (Costar Spin-X columns, Fisher Scientific International). The sample/bead mixtures are then centrifuged and the filtrate used for analysis. Confirmation of reduction of background by this technique was achieved by comparing results with antibody extraction to results obtained without antibody removal (data not shown).

For the ELISA, clear flat-bottom 96-well microtiter plates are coated with UI-1021 and blocked with StartingBlock. Test calibrators and samples were added to duplicate wells, followed by equal volumes of anti-feline proBNP 100–106 in the sample buffer. The plate was incubated overnight at 2–4 °C, and then washed and incubated overnight in goat anti-rabbit alkaline phosphatase-conjugated antibody (Sigma-Aldrich). The following day, the plate was washed and stained with Attophos. The RFUs of each well were measured as before.

Performance characteristics of the ELISA were evaluated based on recommended methods (Koch and Peters, 2001). Synthetic feline proBNP 80–106 was used as the reference material for the experiments. A reference method does not exist for this assay, and therefore the range of proBNP concentrations selected for evaluation was based on the results of a previous study (Sisson et al., 2003).

The analytical range, linearity, and analytical sensitivity of the ELISA were determined from a test calibration curve made by diluting proBNP 80–106 spiked canine plasma with unspiked canine plasma. Recovery of the sample was tested by spiking four feline plasma samples with a final concentration of 10.5 pmol/L synthetic proBNP 80–106, estimated from a previous study to be near the lower limit of concentrations that might be observed in cats with symptomatic cardiomyopathy (Sisson et al., 2003).

Canine and feline plasma spiked with proBNP 80–106 and serially diluted with unspiked canine and feline plasma, were used to test the effect of the test calibrator matrix on reported values. Within run precision was tested using pooled feline plasma spiked with two concentrations of proBNP 80–106, and the test was repeated 10 times on each sample in the same test run to calculate the percent coefficient of variation (CV). Intra-assay precision between duplicate test wells was calculated using the results obtained from the feline patients described below.

The cross reactivity of the proBNP ELISA was tested against canine, human, and rat C-terminal proBNP, and C-terminal ANP 5–28 and CNP 22. The ability of a protease inhibitor mixture to preserve proBNP 80–106 in feline plasma was tested using pooled cat plasma spiked with proBNP 80–106. The sample was split, and protease inhibitors were added to one aliquot in the following final concentrations: 10 μg/mL PPACK II, 20 μg/mL aprotinin, 200 μg/mL benzamidine, and 100 μg/mL leupeptin hemisulfate (Sigma-Aldrich). The protease inhibitor-treated and untreated plasma were incubated side-by-side at 2–4 °C for 5 days. Aliquots from the stored samples were removed after 1, 2 and 5 days, and frozen at –80 °C. After collection of the final aliquot, the feline proBNP ELISA was performed in duplicate in a single run.

Table 1
Comparative amino acid sequences of natriuretic peptides through region of monoclonal antibody UI-1021. Shaded regions denote homologous amino acid sequences to feline proBNP.

	Feline proBNP amino acid number											
Peptide	84	85	86	87	88	89	90	91	92	93	94	95
Cat proBNP	C	F	G	R	R	L	D	R	I	G	S	L
Dog proBNP	C	F	G	R	R	L	D	R	I	G	S	L
Human proBNP	C	F	G	R	K	M	D	R	I	S	S	S
Rat proBNP	C	F	G	Q	K	I	D	R	I	G	A	V
Human CNP	C	F	G	L	K	L	D	R	I	G	S	M
Human ANP	C	F	G	G	R	M	D	R	I	G	A	N

Detection of proBNP in feline plasma

The C-terminal proBNP concentration in feline plasma was measured using plasma from privately-owned cats that presented to the Matthew J. Ryan Veterinary Hospital of the University of Pennsylvania for evaluation of possible cardiac disease, or that were healthy cats owned by hospital staff or students. The collection of plasma samples was approved by the University of Pennsylvania Institutional Animal Care and Use Committee (Protocol 801710, 2 December 2008).

All cats received a physical examination and cardiac ultrasound. In each cat, the cardiac diagnosis (i.e., healthy vs. cardiomyopathy) and clinical class of heart disease were determined and recorded using the International Small Animal Cardiac Health Council (ISACHC, 1999) system. Briefly, the ISACHC classes describe cats that are healthy (ISACHC 0), cats with asymptomatic heart disease (ISACHC 1) with (1b) or without (1a) cardiac enlargement, cats with mild heart failure (ISACHC 2), and cats with severe heart failure (ISACHC 3a and 3b). The cardiac status of each cat was determined before the results of the BNP analyses were known.

Blood for analysis was collected into EDTA Vacutainer tubes containing the protease inhibitor mixture. Plasma was harvested from each blood sample and stored frozen until analysis.

Statistical methods

Statistical tests were done using GraphPad StatMate and Prism version 6 software. An r^2 value of >0.9 was accepted as evidence of goodness of fit of linear regression lines. Statistically significant P values were defined as ≤0.05. The mean and 95% confidence intervals (CI) of K_{aff} were used to determine the acceptability of UI-1021 as a capture antibody for feline C-terminal proBNP. Linear regression, a sample run post-test, and visual inspection of the ELISA standard calibration curve were used to assess the test's analytical range, linearity, and analytical sensitivity. Spike recovery was calculated by first subtracting the baseline proBNP 80–106 concentration from the measured proBNP concentration. The average percent recovery and 95% CI of the spike in each of the four plasma samples were then calculated by dividing the amount of spike recovered by the amount added. Parallelism between results obtained using feline plasma or canine plasma as sample matrix was tested by comparison of the slopes and y-intercepts of linear regression lines of serial dilutions of proBNP 80–106 in both plasma types.

Determination of the necessary minimum sample size of the two groups (cardiomyopathy and healthy) for the clinical portion of the study was done by statistical power analysis. The anticipated means and standard deviations of plasma C-terminal proBNP concentrations that would be observed in the two groups were based on the results of a prior study (Sisson et al., 2003). It was calculated that a minimum of approximately 10 cats per group would be necessary to detect a difference between the two groups with a power of 95%.

The one-tailed Mann–Whitney test was used to compare C-terminal proBNP concentrations between healthy cats (ISACHC stage 0) and cats with cardiomyopathy, and to compare cats with asymptomatic cardiomyopathy (stages 1a and 1b) to symptomatic heart failure stages (stages 2, 3a and 3b). Normality of the C-terminal proBNP values from the healthy cats and cats with cardiomyopathy was tested with D'Agostino and Pearson omnibus test.

Results

The calculated mean K_{aff} of UI-1021 was 2.58×10^9/M (95% CI = 1.36×10^9/M to 3.81×10^9/M). Based on the assumption that the K_{aff} for antibodies used in immunoassays should be 10^8/M or greater (Davies, 2001) the affinity of UI-1021 was deemed adequate. Epitope linear mapping identified a four-residue antibody binding motif (CFGR) near the N-terminal portion of the natriuretic peptide ring (feline proBNP 84-87).

The proBNP 80–106 ELISA calibration curve was linear throughout an analytical range of 1.4–85 pmol/L ($r^2 = 0.9949$), with an analytical sensitivity of 1.4 pmol/L (Fig. 2). Serial dilutions of proBNP 80–106 in feline and canine plasma revealed no significant difference in either the slopes or y-intercepts ($P = 0.381$ and $P = 0.1977$, respectively) of the respective regression lines. A sample matrix effect that might require correction of values generated from a calibration curve made using canine plasma was therefore not detected. Sample recovery averaged 106.5% (95% confidence interval = 78–135%) with measured values ranging from 9.3 to 13.1 pmol/L. The within run CV was 11.9% at a mean concentration of 4.3 pmol/L, and 5.1% at a mean concentration of 13.2 pmol/L. Intra-assay CV ranged from 0.01% to 8.6%, and averaged 2.4%.

The feline proBNP ELISA did not immunoreact with canine, human, or rat C-terminal proBNP, or with C-terminal ANP 5–28 or CNP 22. Feline proBNP 80–106 immunoreactivity was retained throughout 5 days of storage in the presence of PI mixture but was lost within 1 day without the PI mixture (Fig. 3).

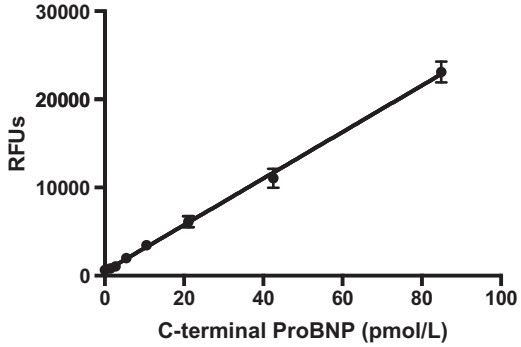

Fig. 2. Calibration curve of feline proBNP 80–106 in canine plasma revealed the test to be linear throughout its analytical range.

Thirty-one privately owned cats, including 18 healthy cats and 13 cats with cardiomyopathy, were recruited for C-terminal proBNP blood testing. All of the cats in the study were either domestic longhair or shorthair breeds. The median age of the healthy cats was 3 years (range 1–10 years). The median age of the cats in the cardiomyopathy group was 9 years (range 1–15 years). All cats in the healthy group (8 males, 10 females) and in the cardiomyopathy group (10 males, 3 females) were neutered.

The diagnoses and the number of cats with each form of cardiomyopathy were: hypertrophic or hypertrophic obstructive cardiomyopathy ($n = 8$), restrictive or unclassified cardiomyopathy ($n = 4$), and moderator band cardiomyopathy ($n = 1$). The number of cats in each ISACHC category was: 1a ($n = 1$), 1b ($n = 6$), 2 ($n = 2$), 3a ($n = 1$), and 3b ($n = 3$). The plasma concentrations of C-terminal proBNP in the cats without heart disease were ≤1.8 pmol/L, while the proBNP values in the cats with cardiomyopathy were significantly higher ($P = 0.0001$), and ranged from 1.7 to 78.8 pmol/L, with a median value of 15.6 pmol/L (Fig. 4). The data were not normally distributed in either group.

The median proBNP concentration of cats with asymptomatic cardiomyopathy (ISACHC stages 1a and 1b) was 11.4 pmol/L (range = 1.7–36.8 pmol/L), and significantly less ($P = 0.0256$) than C-terminal proBNP concentrations in the cats with symptomatic ISACHC stages 2, 3a and 3b disease, where the median concentration was 24.6 pmol/L (range 10.1–78.2 pmol/L).

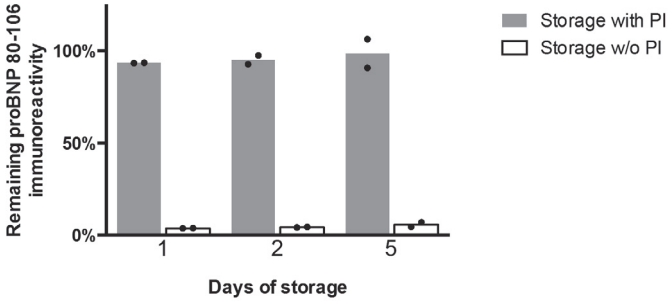

Fig. 3. Stability of feline C-terminal proBNP immunoreactivity with storage. Pooled feline plasma spiked with proBNP 80–106 was stored for up to 5 days at 2 °C–4 °C with or without a mixture of protease inhibitors (PI). The individual points show the measured concentrations in each sample, which were run in duplicate.

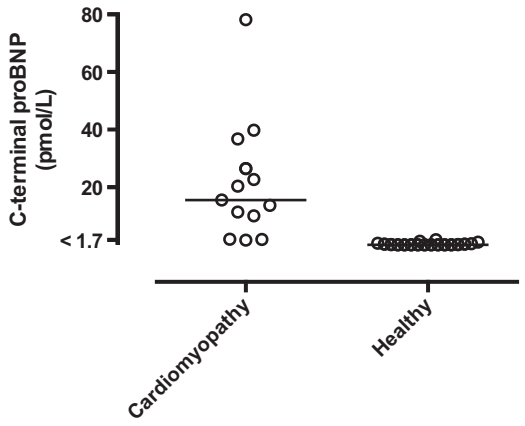

Fig. 4. Plasma C-terminal proBNP concentrations from 13 cats with cardiomyopathy and 18 healthy control cats. Bars show median values.

Discussion

The low-picomolar concentrations of plasma C-terminal proBNP found in this study are similar to plasma BNP concentrations reported in human patients (Balion et al., 2006) and canine patients (DeFrancesco et al., 2007). The concentrations are slightly less than those observed by our group previously using a canine competitive radioimmunoassay with cross reactivity to the feline C-terminal 17-amino acid ring (Sisson et al., 2003). Although a variety of factors could be responsible, the higher values in the former study are consistent with what would be predicted given that the sandwich ELISA requires two intact antigenic sites as opposed to one, making the sandwich ELISA more susceptible to the effects of degradation of C-terminal proBNP. The natriuretic peptides are susceptible to processing by several proteases in plasma (Kenny et al., 1993; Lam et al., 2007; Gomez et al., 2012; Ichiki et al., 2013; Huntley et al., 2015), and post-sampling peptide degradation can be a challenge to the analysis of BNP (Connolly, 2010). However, this study showed that the protease inhibitor mixture used was effective, and therefore the measured proBNP concentrations are considered an accurate reflection of the blood concentrations at the time of sampling.

Since the binding site of UI-1021 encompasses the area of hydrolysis of proBNP by neprilysin, the protease believed primarily responsible for the inactivation of circulating natriuretic peptides (Kenny et al., 1993; McKie and Burnett, 2015), this assay probably doesn't detect BNP inactivated by this major mechanism. However, it is known that human heart failure patients have uncleaved proBNP as well as fragmented forms of BNP with degraded N-terminal segments in the circulation (Miller et al., 2011), and little or none of what is typically measured consists of appropriately cleaved, not degraded BNP (Hawkridge et al., 2005). Whereas the two antigenic sites used in the ELISA of this study would be present in these alternate forms, it may be possible that the concentrations of biologically active BNP implied by our results overestimate the true concentrations in the circulation of cats with cardiomyopathy. Tests measuring uncleaved proBNP or degraded forms of BNP have yet to be reported in cats.

The current findings also show that circulating feline plasma proBNP immunoreactivity includes peptides with an intact C-terminus portion (proBNP 100–106), and that the circulating concentrations of C-terminal peptide are increased in cats with cardiomyopathy as compared with concentrations in the plasma of healthy cats. Plasma C-terminal proBNP concentrations were also found to be greater in cats with symptomatic cardiomyopathy than in asymptomatic cardiomyopathy, as was observed in an earlier study (Sisson et al., 2003). However, the plasma concentrations of C-terminal peptide observed in this study were <10% of the N-terminal proBNP concentrations reported in other studies (Connolly, 2010). Relatively small C-terminal peptide concentrations have also been observed in human heart failure patients and are believed to reflect the clearance of the active peptide by natriuretic peptide receptors, which do not exist for N-terminal peptide (Arteaga et al., 2005; Balion et al., 2006; Masson et al., 2006; Waldo et al., 2008; Macheret et al., 2011).

A decrease in the ratio of plasma N-terminal proBNP to uncleaved proBNP with advancing stage of heart failure has also been observed in human patients, the speculated cause of which is either decreased secretion or increased peripheral processing of uncleaved proBNP (Macheret et al., 2011). The plasma concentrations of N-terminal proBNP were not measured in this study, and it has yet to be determined whether feline cardiomyopathy is associated with a similar occurrence. Nevertheless, the similarities between the feline and human proBNP systems, including the absolute amounts of N-terminal and C-terminal peptides, along with the low picomolar concentrations of the latter, make it possible that, as in humans, feline CHF might be a state of relative BNP deficiency.

It has been proposed that deficiencies of biologically active BNP contribute to the BNP paradox, the progression of CHF, and is a principal rationale for exogenous BNP treatment (Chen, 2007; Liang et al., 2007; Huntley et al., 2015; McKie and Burnett, 2015). If further work is similarly indicative of cats experiencing a relative BNP deficiency in heart failure then it is possible that cats may also benefit from BNP therapy.

Conclusions

This study confirms that C-terminal proBNP is present in feline plasma in low picomolar concentrations, which are substantially less than reported N-terminal proBNP concentrations. These findings are similar to findings in human heart failure patients, where relative deficiencies of biologically active BNP have been proposed as a contributing cause of the BNP paradox. It is anticipated that the antibodies and ELISA developed for this study will be useful in enhancing an understanding of the feline natriuretic peptide system in cats with cardiomyopathy.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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