Clinical Use of N-terminal Pro-Brain Natriuretic Peptide Concentrations for Assessing the Severity and Prognosis of Myxomatous Mitral Valve Disease in Dogs

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ABSTRACT

We have evaluated N-terminal pro-brain natriuretic peptide (Nt-proBNP) as a diagnostic and prognostic plasma biomarker in dogs with asymptomatic and symptomatic myxomatous mitral valve disease (MMVD). One hundred and twenty-nine dogs with MMVD and 61 healthy dogs were followed from August 2006 to May 2008. Clinical end points were death or progression of the disease. Nt-proBNP concentrations were found to be significantly higher in dogs with MMVD than in control dogs. In dogs with symptomatic MMVD, survival analysis showed a shortened median event-free period in the group having higher concentrations of Nt-proBNP (134 days) compared to the group with lower concentrations of Nt-proBNP (669 days). Heart rate and Nt-proBNP levels were identified as independent prognostic risk factors for progression of heart failure in dogs with symptomatic MMVD. Our results suggest that the measurement of Nt-proBNP concentration is a diagnostic and prognostic biomarker in dogs with symptomatic MMVD.

INTRODUCTION

Brain natriuretic peptide (BNP) is a peptide hormone secreted from the ventricular myocardium in response to stretch or neurohormonal activation. This prohormone is enzymatically cleaved into biologically-active BNP and the inactive fragment Nt-proBNP. The longer half-life of Nt-proBNP supported clinical application as biomarker

of heart disease severity. The concentration of circulating Nt-proBNP was shown to increase proportionally with the left ventricular end-diastolic pressureand left ventricular end-diastolic wall stress.[1-3]Accordingly, NT-proBNP is a sensitive and predictive marker of cardiac disorders in the dog.[4,5]

Current efforts are directed at the potential of Nt-proBNP for the detection of early signs of heart failure. This proactive approach could potentially lead to successful treatment outcomes. A single study evaluated the capacity of Nt-proBNP to detect asymptomatic MMVD in a small number of Cavalier King Charles Spaniels with moderate to severe mitral regurgitation, and reported inconclusive results.[6] The aim of the present work was to clarify the clinical potential of Nt-proBNP as a diagnostic tool for the detection of asymptomatic MMVD using a controlled longitudinal study design including a 19-month follow-up of the plasma Nt-proBNP levels in healthy and MMVD dogs.

CASES AND METHODS

Cases

The study population consisted of client-owned dogs that presented with a left apical systolic murmur (grade > 2) and healthy beagle dogs. The subjects for the study protocol were cared for in accordance with the guidelines for the care and use of laboratory animals approved by the College of Bioresource Science, Nihon University. Owner consent was obtained for all dogs included in the study. Two veterinary cardiology practices recruited the dogs between August 2006 and May 2008. Dogs were eligible for inclusion in the study if the diagnosis of MMVD could be confirmed by echocardiographic detection of thickened leaflets, prolapsed mitral valve leaflets, rupture of the chordae tendinae, and evidence of mitral regurgitation with color-flow Doppler.

Congenital heart disease and acquired heart disease other than MMVD or systemic disease were excluded from the study. Dogs with MMVD and tricuspid regurgitation and dogs with prerenal azotemia associated with

MMVD were included in the study. Healthy dogs were considered on the basis of physical examinations and blood tests as complete blood count, total protein, albumin, urea nitrogen, creatinine, alkaline phosphatase, alanine aminotransferase, and aspartate amino transferase.

Assessment of Cardiac Disease Severity

Venous blood was collected from all dogs, and all of them underwent a complete physical examination that included cardiac auscultation. Blood samples were obtained from the jugular or cephalic vein and immediately placed in a tube containing potassium-EDTA as an anticoagulant. Samples were centrifuged within 30 minutes after collection at 3,000 rpm for 10 min, and the supernatant (plasma) was transferred to a plastic tube and stored at -80° C. All dogs with MMVD underwent thoracic radiography and 2-D, M-mode, and color-flow Doppler echocardiography. Standard echocardiographic techniques.[7,8] were used to measure the dimensions of the left ventricle at end-diastole (LVIDd) and end-systole (LVIDs), the aorta, and the left atrium. In dogs with MMVD, the severity of heart failure was classified according to the International Small Animal Cardiac Health Council (ISACHC) recommendations based on clinical symptoms and the thoracic radiographsat the first examination.[9]

Measurement of Nt-proBNP

Plasam Nt-proBNP concentration was measured with a commercial enzyme immunoassay kit using antibodies raised against canine Nt-proBNP according to the manufacturer's instructions (Canine Cardioscreen, VET-SIGN, Guildhay). The kit incorporates two immunoaffinity purified sheep antibodies specific for canine Nt-proBNP. The plate consists of a capture antibody, anti-Nt-proBNP (25-41), bound to the wells of the plate. The tracer contains the detection antibody, anti-Nt-proBNP (1-22), conjugated to horseradish peroxidase. Nt-proBNP was detected colorimetrically at a wavelength of 450 nm. Five serum samples with known Nt-proBNP concentrations were measured for quality

control purposes every time the Nt-proBNP assay was performed. Samples were assayed in duplicate, and the mean value of the two measurements was used for all analyses.

Follow-up

Clinical evaluations were performed at least monthly throughout the follow-up period. The primary end point was death due to the aggravation of heart failure or an increase in the severity of the ISACHC class. Dogs lost from the study during the follow-up period and those that died of causes other than cardiac disease were not included in the statistical analysis. Dogs that remained alive were censored at the end of follow-up.

Statistical Analysis

Statistical values are presented as the median and the interquartile range (IQR, 25th to 75th percentile). The Friedman test was used to compare plasma Nt-proBNP concentrations between intact male, neutered male, intact female, and neutered female healthy dogs. Correlations between age or body weight (BW) and plasma Nt-proBNP concentration were examined by Pearson's correlation coefficient test.

The Mann-Whitney's U test was used to compare plasma Nt-proBNP concentrations between control dogs and dogs with each ISACHC classification of MMVD. The Kruskal-Wallis test followed by the Dunn multiple comparison test was used to compare plasma Nt-proBNP concentrations among dogs in each ISACHC class. Receiver operating characteristic (ROC) analysis was performed to determine optimal cut-off values for plasma Nt-proBNP concentration in discrimination between dogs with MMVD and control dogs. ROC curves were drawn by plotting all the sensitivity values against their corresponding 1-specificity values. The area under the ROC curve and the 95% confidence intervals (CI) of the prediction of the area were calculated.

Pearson's correlation coefficient test was performed to examine correlations between plasma Nt-proBNP concentration and age, BW, heart rate, the left atrium to aortic root ratio (LA/Ao), the end-diastolic left ven-

tricle internal diameter to BW ratio (LVIDd/BW), the end-systolic left ventricle internal diameter to BW ratio (LVIDs/BW), and fractional shortening (FS). The Spearman rank correlation was calculated to assess the correlation between plasma Nt-proBNP concentration and heart murmur grade. Stepwise multiple regression analysis was performed to identify continuous variables associated with plasma Nt-proBNP concentration.

Survival curves were estimated according to the Kaplan-Meier method and compared using the log-rank test. The association of independent variables with time to outcome was assessed by Cox regression and is expressed as the hazard ratio (HR) and 95% CI.

All analyses were performed with standard software. Values of P < 0.05 were considered significant.

RESULTS

Sixty-one healthy control dogs were included in the study. Sex distribution (20 sexually intact males, 12 castrated males, 13 sexually intact females, and 16 spayed females) did not differ significantly among groups. The breeds consisted of 21 Miniature Dachshunds; 7 Toy Poodles; 6 Welsh Corgis; 3 each of American Cocker Spaniels, Chihuahuas, Jack Russell Terriers, and Papillons; 2 each of Cavalier King Charles Spaniels, Golden Retrievers, mixed breeds, and Shibas; and 1 Beagle, Border Collie, French Bulldog, Labrador Retriever, Maltese, Miniature Schnauzer, and Yorkshire Terrier. There was no significant difference in age (median, 64 months; IQR, 35 to 94 months) or BW (median, 6.7 kg; IQR, 4.7 to 10.9 kg) among groups. The median plasma Nt-proBNP concentration in control dogs was 243.7 pmol/L (IQR, 179.9 to 379.3 pmol/L; minimum, 80.2 pmol/L; maximum, 831.9 pmol/L). There were no significant differences between males (median, 232.3 pmol/L; IQR, 165.9 to 395.9 pmol/L) and females (median, 246.4 pmol/L; IQR, 199.7 to 346.4 pmol/L). Castration did not affect plasma Nt-proBNP concentration as there was no significant variation in the plasma

Nt-proBNP concentration among groups (intact male: median, 284.8 pmol/L, IQR, 191.5 to 372.0 pmol/L; neutered male: median, 232.3 pmol/L, IQR, 165.9 to 415.1 pmol/L; intact female: median, 205.2 pmol/L, IQR, 199.7 to 331.0 pmol/L; neutered female: median, 290.0 pmol/L, and IQR, 198.9 to 353.9 pmol/L). There was no correlation between plasma Nt-proBNP concentration and age or BW in control dogs.

In total, 129 dogs with MMVD were enrolled in the study. The study population was mostly composed of male (n = 82, 63.6%), adult (median, 131 months; IQR, 108 to 146 months), and small-sized dogs (median, 6.6 kg; IQR, 4.2 to 8.4 kg). More than 50% of the dogs were asymptomatic (ISACHC class I), and the different classes of symptomatic MMVD were about equally represented (Table 2).

All dogs exhibited a systolic murmur that was characterized as either an early systolic murmur or as a holosystolic murmur (median heart murmur grade, 3; IQR, 3 to 4). Sixty-five dogs (50.4%) were classified as asymptomatic or ISACHC class I, 26 (20.2%) were ISACHC class II, and 38 (29.5%) were ISACHC class III. In four dogs suffering from ISACHC class IIIb MMVD, echocardiographic examination was not performed for fear of causing severe pulmonary edema. Baseline values of continuous variables for the 129 dogs at first examination are summarized in Table

Table 1. *Baseline characteristics of the study population including 129 dogs with myxomatous mitral valve disease. The median and interquartile values are shown.*

Variable	ISACHC				
	Ia (n = 41)	Ib (n = 24)	II (n = 26)	IIIa (n = 24)	IIIb (n = 14)
Age (months)	118 [75-138]	127 [113-141]	140 [115-164]	138 [121-164]	134 [108-146]
Body weight (kg)	6.3 [4.4-8.4]	7.0 [5.5-8.4]	7.2 [5.1-9.3]	4.8 [3.9-8.1]	7.3 [5.5-8.3]
Heart murmur grade	3 [2-3]	3 [3-4]	4 [3-4]	4 [4-5]	5 [4-5]
Heart rate (beats/minute)	120 [108-144]	123 [117-137]	130 [103-144]	153 [135-168]	146 [132-173]
LA/Ao	1.4 [1.3-1.6]	1.8 [1.7-2.1]	2.0 [1.7-2.3]	2.5 [2.4-2.7]	2.3 [2.1-2.9] (n = 10)
LVIDd/BW	4.5 [3.2-5.4]	4.7 [3.7-5.7]	4.3 [3.6-6.3]	6.2 [5.2-9.2]	5.6 [5.3-6.0] (n = 10)
LVIDs/BW	2.3 [1.9-3.3]	2.7 [2.2-3.2]	2.1 [1.8-3.3]	3.5 [2.3-4.0]	2.8 [2.5-3.6] (n = 10)
Fractional shortening (%)	44.8 [38.0-50.9]	46.5 [40.9-50.9]	50.0 [45.8-53.5]	52.7 [41.9-59.8]	47.3 [42.4-50.3] (n = 10)

Data are given as medians and 25-75th percentiles.
ISACHC = International Small Animal Cardiac Health Council; n = number of dogs for which variables were available; LA/Ao = left atrium to aortic root ratio; LVIDd = left ventricle internal diameters in diastole; LVIDs = left ventricle internal diameters in systole; BW = body weight.

Table 2. *Sensitivity and specificity of plasma N-terminal pro-brain natriuretic peptide concentration to differentiate dogs with myxomatous mitral valve disease from control dogs.*

Population considered	Area under ROC curves	95% CI	Cut-off points (pmol/L)	Sensitivity	Specificity
Whole population (n = 129)	0.864	0.813-0.915	> 247.0	0.95	0.54
			> 433.8	0.71	0.85
			>871.9	0.47	1
ISACHC class I (n = 65)	0.755	0.670-0.840	> 247.0	0.89	0.54
			> 433.8	0.46	0.85
			>871.9	0.08	1
ISACHC class II, III (n = 64)	0.975	0.953-0.997	> 245.8	1	0.52
			> 436.7	0.95	0.85
			>876.2	0.86	1

ROC = Receiver Operating Characteristic; CI = Confidence Interval; ISACHC = International Small Animal Cardiac Health Council.

1. At the beginning of the study period, 82 (63.6%) dogs were receiving treatment for their cardiac disease, 14 dogs were receiving monotherapy with angiotensin converting enzyme (ACE) inhibitor, and eight dogs were receiving monotherapy with carvedilol. Sixty dogs were receiving combination therapy with more than two of the following: ACE inhibitors, diuretics (furosemide, torsemide, spironolactone), carvedilol, digoxin, and pimobendan.

Median plasma Nt-proBNP concentrations in dogs of each ISACHC class were significantly higher than the median plasma Nt-proBNP concentration of control dogs. Median plasma Nt-proBNP concentrations in dogs of ISACHC class II (median, 1432.8 pmol/L; IQR, 650.6 to 1791.2 pmol/L), class IIIa (median, 2457.4 pmol/L; IQR, 2003.4 to 3358.9 pmol/L) and class IIIb (median, 5048.9 pmol/L; IQR, 3567.0 to 5048.9 pmol/L) were significantly higher than those of ISACHC class Ia (median, 337.1 pmol/L; IQR, 286.7 to 533.1 pmol/L) and ISACHC class Ib (median, 532.3 pmol/L; IQR, 406.8 to 611.1 pmol/L) (Figure 1). Additionally, the median plasma Nt-proBNP concentration in dogs with advanced heart failure (ISACHC class IIIa and class IIIb) were significantly higher than those in dogs with mild to moderate heart failure (ISACHC class II).

The characteristics of ROC curves used to discriminate dogs with MMVD from control dogs were measured separately for the whole study population, for dogs with asymptomatic MMVD (ISACHC class Ia and class Ib), and for dogs with symptomatic MMVD (ISACHC class II, class IIIa and class IIIb). The results are presented in Table 2. The highest sensitivity and specificity (71% and 85%) were found for a Nt-proBNP cut-off concentration of 433.8 pmol/L in the whole population. However, the optimal Nt-proBNP cut-off concentrations were different when dogs with asymptomatic MMVD (247.0 pmol/L) and those with symptomatic MMVD (876.2 pmol/L) were measured separately.

Regression analyses were conducted to determine whether abnormally high plasma Nt-proBNP concentration is a predictor of heart disease. Significant positive correlations were obtained with age (r = 0.22, r^2 = 0.05, P < 0.05), heart rate (r = 0.33, r^2 = 0.11, P < 0.001), LA/Ao (r = 0.62, r^2 = 0.38, P < 0.001), LVIDd/BW (r = 0.44, r^2 = 0.19, P < 0.001), LVIDs/BW (r = 0.44, r^2 = 0.19, P < 0.001), and heart murmur grade (r = -1.98, P < 0.01). Furthermore, stepwise multiple regression analysis revealed that LA/Ao

Table 3. *Stepwise multiple regression analysis to determine which parameters were independently associated with N-terminal pro-brain natriuretic peptide concentration.*

Dependent variable	Independent variable	Regression coefficient	Standardized regression coefficient	t	P Value
Nt-proBNP	LA/Ao	1569.1	0.530	7.020	<0.01
($r^2 = 0.400$, P < 0.01)	LVIDs/BW	300.4	0.199	2.633	<0.05

The variables of age, body weight, heart rate, LVIDd/BW, and fractional shortening were eliminated by the stepwise method. Nt-proBNP = N-terminal pro-B-type natriuretic peptide; LA/Ao = left atrium to aortic root ratio; LVIDs = left ventricle internal diameters in diastole; BW = body weight.

and LVIDs/BW could be used to predict the plasma Nt-proBNP concentration (Table 3).

Dogs with symptomatic MMVD (ISACHC class II, class IIIa, and class IIIb) were divided into two groups. One group consisted of dogs with plasma Nt-proBNP concentrations above the median value (2,118.9 pmol/L) and one group consisted of dogs with concentrations less than the median value. Kaplan-Meier survival curves with respect to progression of heart failure in dogs with symptomatic MMVD are illustrated in Figure 2. The dogs with symptomatic MMVD and concentrations of plasma Nt-proBNP above the median had a significantly greater probability of progression of heart failure (median event-free duration, 134 days) compared with those having concentrations less than the median value (median event-free duration, 669 days). Using multivariate Cox analysis, heart rate and Nt-proBNP concentration were found to be independent prognostic factors for progression of heart failure in dogs with symptomatic MMVD. A 10 bpm increase in heart rate increased the hazard of progression of heart failure by 22% (95% CI: 1.06 to 1.40, P < 0.01) in the dogs with symptomatic MMVD. A 100 pmol/L increase in Nt-proBNP concentration increased the hazard of progression of heart failure by 4% (95% CI: 1.02 to 1.07, P < 0.005) in the dogs with symptomatic MMVD.

DISCUSSION

The range of plasma Nt-proBNP concentration in healthy dogs varied widely from 80.2 pmol/L to 831.9 pmol/L. Kellihan et al. have shown that the Nt-proBNP concentration in healthy dogs varies from week to week[10] and these changes are believed to be responsible for the low specificity in dogs classified as ISACHC class Ia and class Ib.

Figure 1. *Box plots illustrating the plasma N-terminal pro-brain natriuretic peptide (Nt-proBNP) concentrations in control dogs and in dogs with myxomatous mitral valve disease (MMVD) grouped on the basis of International Small Animal Cardiac Health Council (ISACHC) class. In each box, the central horizontal line indicates the median value, the box includes the 25th-75th percentiles, the whiskers represent the 5th and 95th percentiles, and circles indicate outliers that lie outside the 5th and 95th percentiles.*
* P < 0.001 compared with Ia, * * P < 0.01 compared with Ib, * * * P < 0.05 compared with Ib, † P < 0.01 compared with II, † † P < 0.05 compared with II.
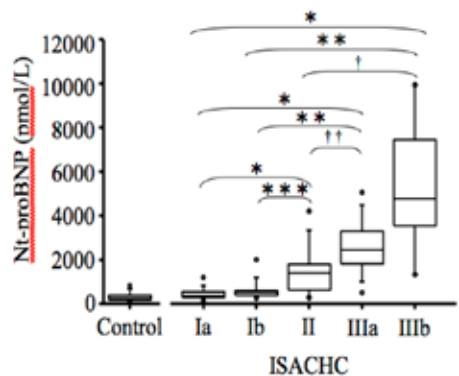
In addition, Tarnow et al. reported that there was no statistical difference in Nt-proBNP concentrations between healthy dogs and Cavalier King Charles Spaniels with no mild mitral regurgitation (regurgitant jet size < 80%).[6] They reported that Nt-proBNP had a sensitivity of 82% and a specificity of 50% at a cut-off value of 299 pmol/L for differentiating healthy dogs from Cavalier King Charles Spaniels with pre-clinical moderate to severe mitral regurgitation.[6] Our results and Tarnow's study both demonstrate that it is difficult to discriminate between healthy dogs and dogs with asymptomatic MMVD based on Nt-proBNP concentration in the plasma.

Since Nt-proBNP is released from the cardiac wall in response to stretch, volume overload should be associated with higher circulating levels of plasma Nt-proBNP. Accordingly, our study shows that plasma Nt-proBNP concentrations were found to be significantly elevated in symptomatic dogs compared to those of asymptomatic dogs. Mitral regurgitation causes an increase in blood volume in the left ventricle leading to an enlargement of this chamber in dogs with heart failure caused by MMVD.[11,12]Hori et al. reported that an artificially induced volume overload increased plasma Nt-proBNP concentrations in healthy dogs.[13] Plasma Nt-proBNP concentrations increased in dogs with heart disease, and this increase was correlated with vertebral heart size, LA/Ao, LVIDd, and LVIDs.[14-16] Our current study in dogs with MMVD is consistent with these earlier reports. All of these results indicate that an increase in Nt-proBNP concentration is associated with cardiac volume overload in dogs with MMVD.

Moreover, the present study demonstrated that

plasma Nt-proBNP concentration was an independent prognostic factor for the progression of heart failure in dogs with MMVD. Multivariate analysis has shown that independent predictors for cardiac-related death in symptomatic MMVD were cardiac enlargement, poor exercise tolerance, and dose of furosemide.[17] Our results suggest that the cardiac biomarker Nt-proBNP is also a useful prognostic indicator for the progression of heart failure in dogs with MMVD in addition to conventional prognostic factors such as physical examination, thoracic radiography, and echocardiography.

Dogs with congestive heart failure caused by MMVD have poor prognoses.[17-19] The median survival time in symptomatic dogs with rupture of the chordae tendineae was 394 days.[20] Furthermore, the BENCH study reported that the mean survival time to reach the next class (ISACHC class III, death or withdrawal) of heart failure in dogs with ISACHC class II was 181 days.[18] Our results, symptomatic dogs (Nt-proBNP concentration > 2118.9 pmol/L) are consistent with the BENCH study in that heart failure

Figure 2. Kaplan-Meier curves of time to progression of heart failure in dogs with myxomatous mitral valve disease (MMVD) of International Small Animal Cardiac Health Council (ISACHC) class II and III divided into 2 groups according to N-terminal pro-brain natriuretic peptide (Nt-proBNP) concentration. Survival in dogs with MMVD and Nt-proBNP > 2118.9 pmol/L differed significantly from those with Nt-proBNP < 2118.9 pmol/L. P < 0.01 by log-rank test for the overall comparison between groups.
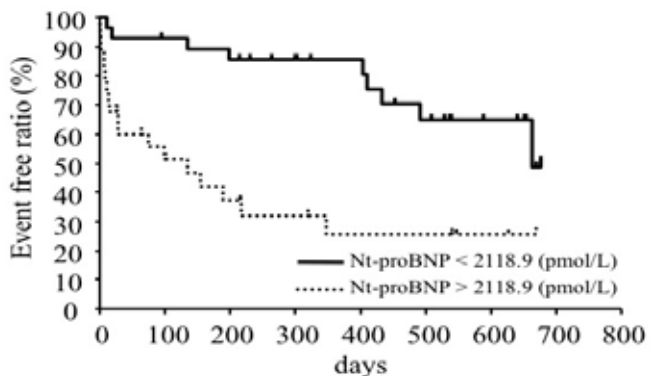
progressed more quickly. These results suggest that heart failure was accelerated and led to early death in dogs with symptomatic dogs. Accordingly, the results of the present study indicate that more intensive monitoring is necessary in dogs with symptomatic dogs and with plasma Nt-proBNP concentrations above 2118.9 pmol/L. Furthermore, surgical mitral valve repair may be indicated in dogs with Nt-proBNP concentrations higher than this value because mitral valve repair improves prognosis in dogs with MMVD.[21,22]

The present study found that heart rate was a prognostic factor for the progression of heart failure in dogs with MMVD. Heart rate is increased by activation of the sympathetic nervous system and by neurohormones, and previous studies have shown that noradrenaline production and heart rates increased in dogs with MMVD as compared to healthy dogs.[23,24]These results suggest that an increased heart rate is indicative of heart failure. An elevated heart rate was also associated with poor prognosis in dogs with rupture of the chordae tendineae.[20] In univariate analysis, heart rates >140 bpm were associated with a survival time decrease in dogs with MMVD,[19] and our results support these previous studies. These findings suggest that heart rate is a useful indicator for progression of heart failure in dogs with MMVD.

There are some limitations in this study. First, 63.6% of the MMVD dogs were treated with medications and these treatments may affect the echocardiography measurements and the Nt-proBNP concentrations. Second, many different breeds were enrolled in the present study. Oyama et al. demonstrated that healthy, purebred dogs had higher Nt-proBNP levels compared with mixed-breed dogs.[14] Thus, the differences in breeds may have affected Nt-proBNP levels.

CONCLUSION

In conclusion, plasma Nt-proBNP concentration is a useful diagnostic biomarker for differentiating dogs with symptomatic MMVD from healthy dogs. Dogs with MMVD and a Nt-proBNP concentration greater than 2118.9 pmol/L are at higher risk of heart failure, and those will be candidate for mitral valve repair.[21,22] We have also shown that Nt-proBNP concentration and heart rate are independent prognostic factors for the progression of heart failure in dogs with MMVD.

Conflict of Interest Statement

The authors do not have any conflict of interest to declare.

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