

## Effect of Heart Failure on Dipeptidyl Peptidase IV Activity in Plasma of Dogs

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**Background:** In congestive heart failure (HF), plasma B-type natriuretic peptide (BNP) seems devoid of biological effectiveness. BNP<sub>1–32</sub> could be truncated into BNP<sub>3–32</sub> by dipeptidyl peptidase IV (DPP4), and BNP<sub>3–32</sub> has reduced biological activities.

**Hypothesis:** Increased DPP4 activity is associated with pathophysiology of HF.

**Animals:** One hundred twenty-eight client-owned dogs and 9 experimental Beagles from the Clinical Veterinary Unit of the University of Liège.

**Methods:** We prospectively measured plasma DPP4 activity in 5 groups of dogs: normal growing dogs (n = 21), normal adult dogs (n = 60), healthy Beagle (n = 9), dogs with myxomatous mitral valve disease (n = 35), and dogs with dilated cardiomyopathy (n = 12). The final diagnosis and the severity of HF were determined by Doppler echocardiography. Plasma DPP4 activity was measured kinetically by a fluorimetric method.

**Results:** In growing dogs, DPP4 activity was higher than in adults ( $P < .001$ ) and inversely correlated with age ( $r = -0.57$ ,  $P < .01$ ). In adults, DPP4 activity increased linearly with body weight ( $r = 0.39$ ,  $P < .01$ ), but there was no influence of age or sex. No effect of the circadian rhythm was noted. DPP4 activity was significantly higher in HF ISACHC I ( $16.3 \pm 1.14$  U/L) compared with healthy adults ( $12.4 \pm 0.65$  U/L,  $P < .05$ ) and HF ISACHC III ( $11.0 \pm 1.50$  U/L,  $P < .05$ ). Mean DPP4 activity in ISACHC II was  $15.1 \pm 1.4$  U/L.

**Conclusion and Clinical Importance:** We did not find evidence that plasma DPP4 activity is responsible for the “BNP resistance” in overt congestive HF, but it may be implicated in early stages.

**Key words:** BNP<sub>3–32</sub>; B-type natriuretic peptide biology; Dilated cardiomyopathy; Myxomatous mitral valve disease.

**I**N overt congestive heart failure (HF), high endogenous B-type natriuretic peptide (BNP) concentrations measured with conventional assays are paradoxically associated with a lack of effect of this hormone. Moreover, several authors have observed that response to treatments targeting the natriuretic peptide system is attenuated in human patients and in dogs with experimental congestive HF compared with healthy animals or animals with mild HF.<sup>1–3</sup> Potential mechanisms of the so-called resistance to natriuretic peptides might include overproduction of

### Abbreviations:

BNP	B-type natriuretic peptide
DCM	dilated cardiomyopathy
DPP4	dipeptidyl peptidase IV
HF	heart failure
ISACHC	International Small Animal Cardiac Health Council
MMVD	myxomatous mitral valve disease

hypertensive hormones counterbalancing the effects of BNP<sub>1–32</sub> but also changes in BNP<sub>1–32</sub> processing,<sup>4</sup> blunted intracellular signaling,<sup>5</sup> and upregulation of “clearance” receptors.<sup>6</sup> Finally, “BNP resistance” might be because of its cleavage into additional truncated forms that are less biologically effective.<sup>7</sup> Obviously, the knowledge of the physiological production, action, and cleavage of BNP<sub>1–32</sub> could influence the way we manage HF. However, this area remains to be investigated.

In vitro, dipeptidyl peptidase IV (DPP4) cleaves BNP<sub>1–32</sub> into BNP<sub>3–32</sub> with higher or comparable efficiency than it does several other known substrates of this enzyme.<sup>8</sup> In dogs, diuretic, natriuretic, and cGMP-generating properties of BNP<sub>3–32</sub> are reduced in contrast to the favorable effects of BNP<sub>1–32</sub>.<sup>7</sup> This observation seems even more clinically relevant when we consider that BNP<sub>3–32</sub> is detectable in human plasma<sup>9</sup> and increased in patients with chronic HF.<sup>10</sup> It would therefore intuitively appear that DPP4 activity could be implicated, at least partially, in the pathophysiology of HF. This study was intended first to determine the physiological determinants of plasma DPP4 activity and second to evaluate plasma DPP4 activity in dogs with HF.

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## Material and Methods

The study protocol was approved by the Committee on Animal Experimentation of the University of Liège. Care to experimental animals conformed to the "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 85-23, National Academy Press, Washington DC, revised 1996).

### Dogs

The study population involved 128 dogs of the Clinical Veterinary Unit of the University of Liège. These included 81 healthy dogs and 47 dogs having a cardiac disease. Nine Beagle dogs belonging to the dog colony of the Clinical Veterinary Unit were used in a substudy to investigate the circadian rhythm effect. Dogs were considered healthy if, as reported by the owners, they did not have a history of clinical disease with systemic repercussions and had a normal physical examination. However, further diagnostic tests to establish their state of health were not carried out. In dogs with cardiac disease, diagnosis and stage of HF were made on the basis of history, clinical signs and consistent results of physical examination, thoracic radiography, ECG, and Doppler echocardiography. Dogs with a systemic disease of noncardiac origin were excluded. Myxomatous mitral valve disease (MMVD) diagnosis was based on remodeling of mitral valve leaflets, mitral valve prolapse, and the presence of a systolic regurgitant flow. Dilated cardiomyopathy (DCM) was diagnosed using proposed guidelines.<sup>11</sup> Dogs with HF were then classified in class I, II, or III according to the International Small Animal Cardiac Health Council (ISACHC) classification system.<sup>12</sup>

### Blood Sampling

For each dog, a 5-mL blood sample was collected into an EDTA tube by jugular venipuncture. To test the circadian rhythm, a venous sample was taken every 6 hours during 24 hours in 9 Beagle dogs. The tubes were centrifuged (10 minutes  $1,200 \times g$ ) at room temperature within 30 minutes of collection. Plasma was transferred to cryotubes and frozen at  $-80^{\circ}\text{C}$  until assay.

### Plasma DPP4 Activity

Plasma DPP4 activity was measured in duplicate by using 0.5 mM glycyl-prolyl-4-methoxy- $\beta$ -naphthylamide (Gly-Pro-4-Me- $\beta$ -NA), diluted from a 100 mM Gly-Pro-4-Me- $\beta$ -NA stock solution in DMSO, as a fluorogenic substrate in a 50 mM Tris buffer pH 8.3. DPP4 activity was determined kinetically over 10 minutes at  $37^{\circ}\text{C}$  by measuring the initial velocities of 4-Me- $\beta$ -NA release ( $\lambda_{\text{ex}} = 340 \text{ nm}$ ,  $\lambda_{\text{em}} = 430 \text{ nm}$ ) from the substrate using an Infinite 200 reader (Tecan Group Ltd, Männedorf, Switzerland). Fluorescence intensity was related to a 4-Me- $\beta$ -NA (stock solution: 20 mM in DMSO, stored at  $-20^{\circ}\text{C}$ ) standard curve (0.5–20  $\mu\text{M}$  range) in the same buffer. One unit of enzymatic activity is the amount of enzyme that catalyzes the release of 1  $\mu\text{mol}$  of 4-Me- $\beta$ -NA from the substrate per minute under the assay conditions.<sup>13,14</sup> All plasma samples were analyzed on the same day. The hemolytic samples were analyzed undiluted and diluted 2-fold to detect any interference; no hemolysis interference was noted. A reference plasma sample was measured in each different run; the between run coefficient of variation was 2.7%. In 11 samples, DPP4 activity was measured with and without sitagliptin, a specific DPP4 inhibitor, to test the specificity of the enzymatic activity; sitagliptin did inhibit the assayed activity by  $98 \pm 1\%$ .

## Statistical Analyses

Statistical analysis was performed using SAS software.<sup>15</sup> Statistical significance was set at  $P < .05$ . The Shapiro–Wilk test was used to test the null hypothesis that DPP4 values were a random sample from a normal distribution. The sex ratio in the different populations was tested by Chi.<sup>2</sup>

Difference in DPP4 activity between healthy growing (<12 months) and adult dogs ( $\geq 12$  months) was tested by unpaired Student's *t*-test. An analysis of covariance was used to test the effects of sex and of age and weight covariates on DPP4 activity in healthy dogs, and linearity was checked for the 2 covariates. When no significant fixed effect was found, simple linear regressions were fitted. Circadian rhythm effect was tested by a one-way analysis of variance for repeated measurements.

In adults (healthy dogs and dogs having a cardiac disease), influence of age and body weight covariables and the fixed effects of sex and categories of cardiac diseases (healthy, MMVD, DCM) and of stages of HF (healthy, ISACHC I, II, and III) on DPP4 activity were tested by an analysis of covariance. For significant fixed effect, the mean separation test was performed on all least squares means pairwise comparisons using the Tukey–Kramer adjustment method.

## Results

### Study Populations

One hundred twenty-nine dogs were distributed as follows: 60 healthy adults of different breeds (range 1–15 years), 21 healthy growing puppies (range 1–11 months), and 47 HF dogs. Dogs with HF were grouped as ISACHC I ( $n = 22$ ), ISACHC II ( $n = 13$ ), and ISACHC III ( $n = 12$ ). Thirty-five dogs were diagnosed with MMVD and 12 dogs with DCM (Table 1).

The DPP4 activity values were normally distributed in the 3 populations of HF dogs and the growing puppies population ( $P > .05$ ), but the healthy adult dog population departed moderately from normality ( $P = .0326$ ).

Dogs with MMVD were older ( $11.3 \pm 0.5$  years old) than healthy adults ( $5.9 \pm 0.4$  years old,  $P < .001$ ) and dogs with DCM ( $7.4 \pm 0.7$  years old,  $P < .001$ ). Dogs with MMVD had a lower body weight ( $10.9 \pm 1.3 \text{ kg}$ ) than healthy dogs ( $21 \pm 1.7 \text{ kg}$ ,  $P < .001$ ) and dogs with DCM ( $35.9 \pm 4.4 \text{ kg}$ ,  $P < .001$ ). Healthy dogs were also lighter than DCM dogs ( $P < .001$ ). There was no significant difference in the sex ratio between healthy dogs and dogs with different stages of HF and between healthy dogs and dogs suffering from a cardiac disease (MMVD or DCM). The echocardiographic characteristics of all HF dogs used in this study are summarized in Table 2. Because these variables were used to construct the groups, we did not compare the groups statistically.

### Plasma DPP4 Activity in Healthy Dogs

In growing dogs, DPP4 activity was significantly higher than in adults (Fig 1A). The analysis of covariance showed no significant sex and weight effects on DPP4 activity in healthy dogs but significant effect for age ( $P < .001$ ) and for this latter

**Table 1.** Demographic data of dogs.

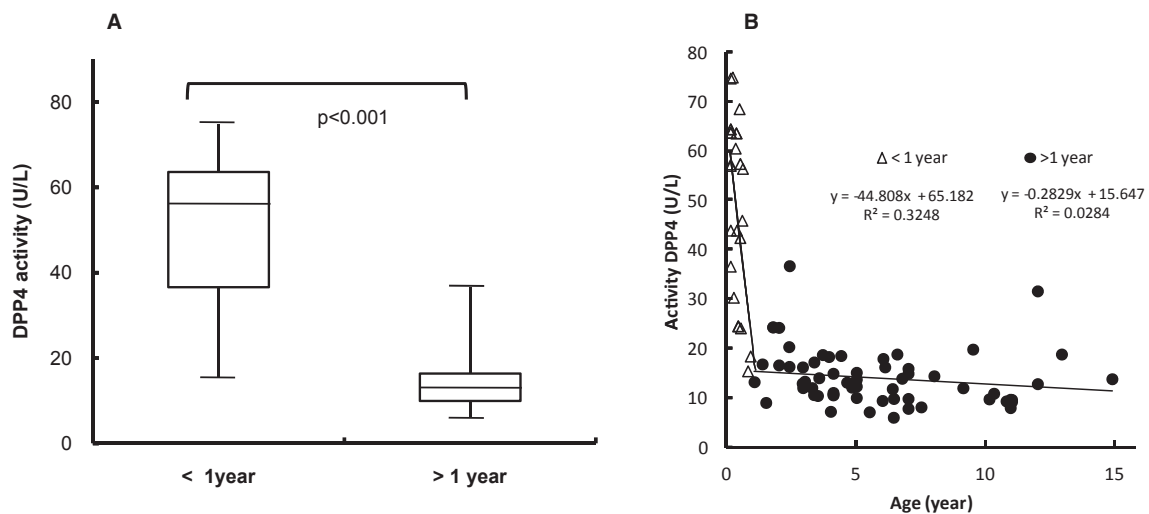
	Healthy		Heart Failure		
	Adult Dogs	Growing Dogs	ISACHC I	ISACHC II	ISACHC III
Number	60	21	22	13	12
Male/female	31/29	12/9	14/8	6/7	9/3
Age, years	5.0 (1.1–14.9)	0.35 (0.13–0.90)	10.5 (4.6–15.7)	12.3 (3.5–14.5)	9.0 (5.9–16.6)
Body weight, kg	21.4 (2.2–52.0)	9.8 (4.0–32.8)	8.6 (3.3–30.6)	12.3 (5.4–62.0)	13.0 (4.9–43)
MMVD			19	9	7
DCM			3	4	5

MMVD, myxomatous mitral valve disease; DCM, dilated cardiomyopathy; ISACHC, International Small Animal Cardiac Health Council; median (range).

**Table 2.** Echocardiographic data of dogs with heart failure.

	DCM				MMVD			
	ISACHC				ISACHC			
	All (n = 12)	I (n = 3)	II (n = 4)	III (n = 5)	All (n = 35)	I (n = 19)	II (n = 9)	III (n = 7)
LVIDd, mm	58 ± 4	52 ± 11	63 ± 6	58 ± 5	38 ± 2	31 ± 3	41 ± 2	46 ± 5
LVIDs, mm	46 ± 4	43 ± 11	49 ± 5	47 ± 3	20 ± 2	17 ± 3	21 ± 2	24 ± 4
FS, %	19 ± 2	18 ± 4	21 ± 2	18 ± 3	49 ± 3	46 ± 5	51 ± 3	51 ± 5
LA/Ao	1.9 ± 0.2	1.7 ± 0.4	1.9 ± 0.2	2.2 ± 0.3	2.0 ± 0.1	1.5 ± 0.1	2.3 ± 0.2	2.7 ± 0.3
LVVId, mL/m <sup>2</sup>	91 ± 8	81 ± 21	106 ± 12	85 ± 10	85 ± 7	64 ± 9	96 ± 13	100 ± 10
LVVIs, mL/m <sup>2</sup>	55 ± 7	51 ± 22	66 ± 5	48 ± 2	21 ± 3	19 ± 3	20 ± 4	28 ± 9
EF, %	41 ± 4	42 ± 9	40 ± 3	41 ± 8	76 ± 2	72 ± 5	81 ± 2	74 ± 7

LVIDd, diastolic left ventricular internal diameter; LVIDs, systolic left ventricular internal diameter; FS, fractional shortening; LA/Ao, left atrial diameter/aortic diameter; LVVId, diastolic left ventricular volume index; LVVIs, systolic left ventricular volume index; EF, ejection fraction; MMVD, myxomatous mitral valve disease; DCM, dilated cardiomyopathy; ISACHC, International Small Animal Cardiac Health Council; mean and standard error of the mean.



**Fig 1.** (A) Plasma DPP4 activity in healthy growing (<1 year old) and adult dogs (>1 year old). DPP4 activity was higher in growing puppies than in adults. The box and whiskers plot demonstrate the median (horizontal lines), 25 and 75% confidence intervals (box), and range (vertical lines). (B) Linear regression for plasma DPP4 activity and age in growing puppies (<1 year) and adult dogs (>1 year).

a significant departure from linearity ( $P < .001$ ). We decided to adjust 2 linear regressions on age, 1 for young dogs less than 12 months of age, and 1 for

adult dogs (>12 months) (Fig 1B). The linear regression was significant for young dogs ( $P < .01$ ). In adult dogs, the linear regression on age was not

significant but was the regression on weight. For this latter, 95% confidence interval was estimated (Fig 2). No marked diurnal change was detected (Fig 3).

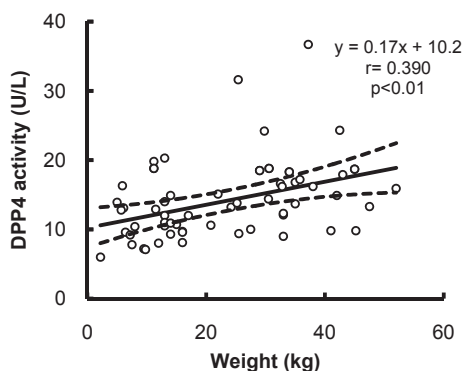
### Plasma DPP4 Activity in Healthy and HF Adult Dogs

Plasma DPP4 activity was significantly influenced by the stage of HF ( $P < .01$ ) and by the body weight ( $P < .01$ ). The pairwise least squares means comparison showed that DPP4 activity was increased in IS-ACHC I ( $16.3 \pm 1.14$  U/L) compared with controls (healthy adults:  $12.4 \pm 0.65$  U/L,  $P < .05$ ) and IS-ACHC III ( $11.0 \pm 1.50$  U/L,  $P < .05$ ) (Fig 4). Mean DPP4 activity in ISACHC II was  $15.1 \pm 1.4$  U/L. Mean DPP4 activity was  $17.1 \pm 1.86$  U/L in DCM and  $13.4 \pm 1.25$  U/L in MMVD, but the difference was not significant.

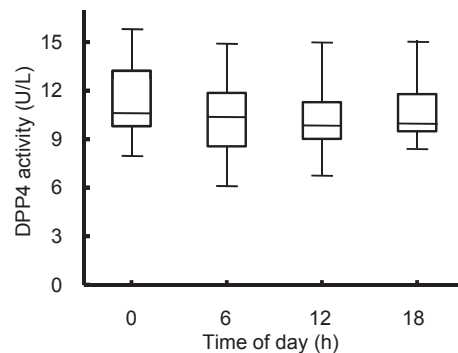
### Discussion

In the present study, we showed that physiological determinants of plasma DPP4 activity in dogs are growth and body weight. Furthermore, we demonstrated that plasma DPP4 activity, an enzyme activity capable of BNP<sub>1-32</sub> truncation, is increased in asymptomatic HF but not in congestive HF.

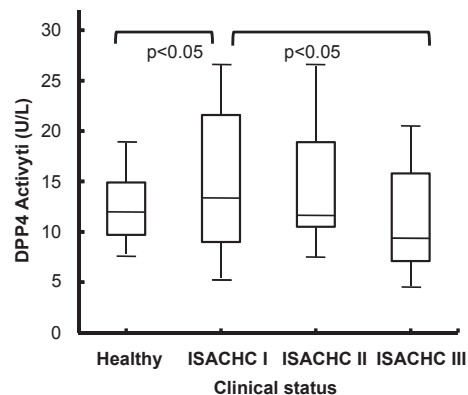
The degradation and elimination mechanisms of BNP are only partly understood.<sup>16</sup> In addition to kidney excretion and internalization by its clearance receptor, BNP<sub>1-32</sub> might be cleared by the neutral endopeptidase but seems relatively resistant to this degradation pathway compared with atrial natriuretic peptide.<sup>17</sup> Endogenous BNP<sub>1-32</sub> undergoes in vivo aminoterminal trimming by an exopeptidase DPP4 also known as adenosine deaminase complexing protein 2 (ADCP 2) or T-cell activation antigen (CD26). This enzyme is a transmembrane protein catalytically active as a homodimer and ubiquitously expressed.<sup>8,9</sup> Its wide tissue distribution and its presence as a soluble active enzyme in body fluids ensure that DPP4 proteolysis is a common event in most



**Fig 2.** Linear regression for plasma DPP4 activity and body weight in healthy adult dogs. Dotted lines represent the 95% confidence interval for predicted values.



**Fig 3.** Plasma DPP4 activity in 9 Beagles during a 24-hour period. There was no marked influence of the circadian rhythm. The box and whiskers plot demonstrate the median (horizontal lines), 25 and 75% confidence intervals (box), and range (vertical lines).



**Fig 4.** Plasma DPP4 activity in healthy adult dogs at different stages of heart failure. The box and whiskers plot demonstrate the median (horizontal lines), 25 and 75% confidence intervals (box), and range (vertical lines).

physiological compartments.<sup>18</sup> DPP4 cleaves dipeptides from the amino terminus of peptides with a proline or alanine in the second position. Human BNP has a proline in the N-terminal penultimate position, and this structure is conserved among several species among which dogs.<sup>8</sup> Canine BNP<sub>1-32</sub> is therefore presumed to be a substrate for DPP4. Up to now, clinically available immunoassays are unable to differentiate between BNP<sub>1-32</sub> and BNP<sub>3-32</sub> in dogs. In view of this fact, we decided to measure DPP4 activity in blood samples of healthy dogs and dogs with HF.

Our 1st objective was to determine the physiological determinants of DPP4 plasma activity. In our study, DPP4 plasma activity was approximately 3.5-fold higher in growing puppies and declined along the growth curve with adult DPP4 activity values being reached around the age of 1 year. To our knowledge, no direct comparison of DPP4 plasma activity between human children and adults is available. But published values in healthy infants are higher<sup>19</sup> than values in adults.<sup>20</sup> In adult humans, as observed in adult dogs,

no association is found between DPP4 activity and age.<sup>20</sup> In adult healthy dogs, we observed an increase of 0.17 U/L/kg of body weight. To our knowledge, in humans, no relationship between size and DPP4 activity has been reported, but there is a positive correlation between DPP4 activity and obesity in humans.<sup>21</sup> Paradoxically, DPP4 activity is depressed in response to poor glycemic control.<sup>22</sup> These topics have not yet been addressed in dogs and merit further investigations. Finally, by using a small number of time points (4/24 hours), we did not detect any marked diurnal change in DPP4 activity.

Our 2nd objective was to measure plasma DPP4 activity in HF dogs as well as the effect of the disease and the stage of HF on its level. We could not find any difference in DPP4 activity between dogs in congestive HF (ISACHC II and III) and healthy dogs. This finding does not corroborate the hypothesis that a higher plasma DPP4 activity is responsible for the "BNP resistance" observed in symptomatic HF. However, future studies should investigate the intracardiac and intravascular DPP4 activity to obtain a complete view of its implication in the pathophysiology of overt congestive HF. On the other hand, DPP4 activity was increased in dogs with asymptomatic (ISACHC I) HF. Data about DPP4 activity in human HF patients have not yet been reported. However, DPP4 activity is increased in plasma of hypertensive patients with latent HF.<sup>23</sup> Moreover, genetic deletion or pharmacological inhibition of DPP4 improves cardiovascular outcomes after myocardial infarction in mice.<sup>24</sup> Whether this cardioprotection was due to the absence of DPP4 activity per se in cardiomyocytes or blood vessels or indirectly due to the subsequent upregulation of cardioprotective molecules could not be inferred from this study. Indeed, DPP4 cleaves not only BNP but also inactivates several other cardioactive peptides, including neuropeptide Y, substance P, stromal cell-derived factor-1, and glucagon-like peptide-1.<sup>18</sup>

A limitation of the study is that the control dogs considered "healthy" did not undergo echocardiography. Therefore, it is possible that some "healthy" dogs had preclinical DCM with no detectable abnormalities on physical examination.

In conclusion, we report that in dogs, plasma DPP4 activity is increased during growth and positively correlated with body weight in adults. Moreover, DPP4 activity is increased in asymptomatic HF but not in congestive HF. Plasma DPP4 activity does not seem responsible for the "BNP resistance" in overt congestive HF but might be implicated in early stages.

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