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ORIGINAL ARTICLE

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Safety of a benazepril and pimobendan combination tablet in adult healthy dogs

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The objective of the study was to investigate the safety of a combination tablet of benazepril and pimobendan, Fortekor PLUS®, in a randomized, blinded, parallel-group design study in healthy adult beagle dogs. The test article, Fortekor PLUS® tablets, was administered orally twice daily for 6 months at one, two, and four times the highest recommended dosage of 0.5 mg/kg benazepril hydrochloride/0.25 mg/kg pimobendan (four males and four females per group). An additional control group was sham-dosed. Fortekor PLUS® did not induce any treatment-related effects on body weight, food consumption, neurological, ophthalmologic or physical assessments over the 6-month treatment period. The test article was possibly associated with an increased frequency of occasional vomiting. Fortekor PLUS® was associated with small, but significant, increases in heart rate and reductions in PR and QT intervals, which were assessed by electrocardiography. These effects were most probably related to reflex tachycardia secondary to reduced systemic blood pressure. Statistically significant changes in some clinical pathology variables were noted after test article administration, but were considered to be of no clinical relevance as values remained within reference ranges and/or were not dose-dependent. No treatment-related macroscopic or microscopic findings were observed. In conclusion, Fortekor PLUS® tablets were well tolerated in healthy adult dogs when administered at one, two, and four times the highest recommended dosage for 6 months.

1 | INTRODUCTION

Congestive heart failure (CHF) is a common clinical syndrome in dogs that is caused most frequently by acquired chronic valvular heart disease (CVHD) or dilated cardiomyopathy (DCM) (Atkins et al., 2009). In addition to reducing the dog's quality of life, CHF also shortens life expectancy. Surgical approaches for the management of canine acquired heart disease have been developing over recent years, but this field of expertize is still in its relative infancy. The majority of dogs with CHF are therefore currently managed by medical (or pharmaceutical) therapy, in conjunction with appropriate diet and exercise programs, to improve cardiac function, clinical signs, quality of life, and survival.

CHF has a complex pathophysiology and treatment varies according to etiology, stage of heart disease and whether the presentation is acute or chronic. The main therapeutic agents recommended are angiotensin-converting enzyme inhibitors (ACEIs), diuretics, and inodilators. In addition, aldosterone antagonists, beta-blockers, and inotropes are used as needed (Atkins et al., 2009).

"Triple therapy" consisting of a combination of the diuretic furosemide, an ACEI and the inodilator pimobendan was recommended in a consensus statement for dogs that have had an episode of heart failure but can be managed at home (Atkins et al., 2009). More recently, the inclusion of the aldosterone antagonist, spironolactone, as part of a "quad therapy" approach to treatment has become increasingly recognized. As a result, many dogs with CHF need to receive several oral formulations each day. To facilitate treatment administration and compliance, Elanco Animal Health has recently developed an innovative flavored tablet, the first registered fixed dose combination of benazepril and pimobendan (Fortekor PLUS®). Benazepril is an ACEI (Balfour & Goa, 1991; Webb, Miller, Traina, & Gomez, 1990), while pimobendan

is an inodilator which combines vasodilation via inhibition of phosphodiesterase III and inotropy due to calcium sensitization (Boyle & Leech, 2012; Ohte, Cheng, Suzuki, & Little, 1997). Both benazepril and pimobendan are registered in the EU and other countries for the treatment of dogs with CHF. The field efficacy and tolerability of Fortekor PLUS® in dogs with CHF were reported recently (King et al., 2017).

In this study, we report the results of a long-term target animal safety study with Fortekor PLUS® in dogs.

2 | MATERIALS AND METHODS

2.1 | Objective

The objective of the study was to evaluate the safety of Fortekor PLUS® tablets in healthy dogs over an extended time period at the recommended dosage (1×) and at elevated dosages, that is, two times (2×) and four times (4×) the 1× dosage.

2.2 | Study design

The study was a randomized, blinded, parallel-group design, negative-controlled study in healthy adult beagle dogs.

The study was conducted in compliance with OECD principles of Good Laboratory Practice at Charles River Laboratories Preclinical Services Ireland Ltd, Glenamoy, County Mayo, Ireland. The study was performed with reference to guidelines for evaluating the target animal safety of new pharmaceuticals, and to recognized quality assurance standards: VICH Guideline 43 on Target Animal Safety for Veterinary Pharmaceutical Products, July 2008 for implementation in July 2009; FDA CVM Guidance 56, Protocol development guidelines for clinical effectiveness and target animal safety trials, July 2001; FDA CVM Guidance 104, content and format of effectiveness and target animal safety technical sections and final study reports for submission to the division of therapeutic drugs for nonfood animals, July 2001; and Good Laboratory Practice, Principles of the Organization for Economic Co-Operation and Development 1998 [ENV/MC/CHEM/(98)17], OECD Consensus Document ENV/JM/MONO (2002) 9 "The Application of the OECD Principles of GLP to the Organization and Management of Multi-site studies" (25 June 2002), the Good Laboratory Practice Regulations of the EC enacted in Ireland in 1999.

The study protocol was reviewed and approved by the site Ethics Committee and the company Global Animal Welfare officer. The study was designed to use the fewest number of animals possible while being consistent with the objectives of the study and regulatory requirements. Dogs, the target species for the test article, were used as the current state of scientific knowledge did not provide acceptable alternatives, in vitro or otherwise, to accomplish the purpose of the study.

The manuscript was prepared after consultation of the ARRIVE Guidelines Checklist for animal in vivo experiments (Kilkenny, Brown, Cuthill, Emerson, & Altman, 2010).

2.3 | Animal details and groups

A total of 32 adult beagle dogs, aged between 9 months and 44 months of age and weighing between 9.8 and 14.4 kg, were selected and acclimatized to a controlled indoor environment for 2 weeks prior to baseline data collection. These dogs had not previously been involved in any other experimental study. From the acclimatization phase until the end of the in-life phase, a period totaling 194 days, the dogs were housed individually in identical pens. Drinking water was supplied ad libitum, and the dogs were fed once per day in the morning with standard commercially available dog food.

2.4 | Randomization and blinding

Each dog was randomly allocated to one of the treatment groups based on homogeneous distribution of body weight and sex criteria (four males and four females per group). The four groups were the control and three different dosages of Fortekor PLUS® representing 1, 2 or 4× of the upper limit of the recommended therapeutic dosages (Table 1). All personnel involved in recording animal data were blinded to the treatment group allocations and were not involved in administration of the test article.

2.5 | Test article and administration

Benazepril was administered as the hydrochloride (HCl) salt, that is, benazepril.HCl. The recommended (minimum) daily dosages for Fortekor PLUS® in the EU are 0.5 mg/kg body weight for benazepril.HCl and 0.25 mg/kg body weight for pimobendan, both divided into two administrations a day (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/veterinary/002804/WC500195474.pdf, accessed 17 Jan 2017). As the tablets are recommended for a weight band, the range of dosages recommended is 0.25–0.5 mg/kg administered twice daily (BID) for benazepril.HCl

TABLE 1 Treatment groups

Group	Daily dosage (mg/kg body weight)[a]		Multiple of maximal therapeutic dosage in Fortekor PLUS®	Number and sex of dogs
	Benazepril.HCl	Pimobendan		
1	0 (control, sham-dosed)	0 (control, sham-dosed)	0	8 (4 m, 4 f)
2	1.0	0.5	1×	8 (4 m, 4 f)
3	2.0	1.0	2×	8 (4 m, 4 f)
4	4.0	2.0	4×	8 (4 m, 4 f)

m, male; f, female.

[a]the total dosage was divided and administered twice daily (BID).

and 0.125–0.25 mg/kg administered BID for pimobendan. For this safety study, the upper end of the dosage range was selected for the 1× dosage, that is, 1.0 mg/kg daily for benazepril.HCl and 0.5 mg/kg daily for pimobendan (Table 1). Doses for each dog were calculated from body weights measured at baseline in the acclimatization period, and during the experimental phase. Single or multiples of tablets were administered to achieve as close as possible the individual calculated target dose.

Tablets were administered *per os*. A small amount of water was then given and the mouth checked to ensure the tablets had been swallowed. The control animals were sham-treated.

Fortekor PLUS® tablets were administered orally BID for 6 months (days 0 to 179 inclusive) and approximately 12 hr apart; once in the morning approximately 1.5 hr before feeding (to be consistent with the label instructions for pimobendan) and a second time in the evening. On the days of dosing, dogs received their feed approximately 1.5 hr after the first administration in the morning.

2.6 | Veterinary examinations and general health observations

The general health of all dogs was checked and recorded once daily by an animal technician. Observations assessed included physical appearance, behavior, abnormalities of feed and water consumption, and appearance of feces and urine. Full veterinary and neurological examinations were performed prior to treatment (on day -14) and regularly throughout the study (days 14, 29, 60, 88, 116, 144, and 179). On days -6, 89, and 173, an ophthalmological examination was also carried out. Adverse events were defined as any harmful and/or unintended event in animals, whether suspected to be treatment related or not.

2.7 | Vomit check

From study day 0 until study day 179, each animal was observed for evidence of vomiting immediately and approximately 10 min following each dosing.

2.8 | Bodyweight and food consumption determination

Body weights were assessed throughout the study, at least once a week and on the day of necropsy. Food consumption was determined daily from the weight of food offered minus that remaining when the feed was removed before the evening administration of the test article.

2.9 | Clinical pathological and laboratory evaluation

Venous blood samples for the determination of hematological, clinical chemistry, and coagulation variables were collected on days -9, 4, 12, 26, 33, 62, 90, 118, 146, and 174. In addition, dogs were placed into cages on those days for separate collection of freely voided urine and feces. Urinalysis (both morphological microscopic and biochemical) was carried out. Fecal samples were inspected for appearance, and assessed for fecal occult blood.

2.10 | Electrocardiographic examination

Electrocardiogram (ECG) recordings from the Lead II position were made over a minimum 30 s period in each dog, while conscious and lightly restrained, on study days -7, 34/35, 60/61, 88/89, 116, 144, and 172. The ECG traces from each animal were examined by a certified veterinary cardiologist for the following variables: heart rate, P-wave duration, P-wave amplitude, PR interval, QRS duration, QRS amplitude, QT interval, ST segment structure, and T-wave amplitude.

2.11 | Necropsy and histological evaluation

At the end of the study (day 179), the dogs were euthanized with intravenous sodium pentobarbitone. A detailed necropsy examination was carried out on all animals under the supervision of a certified veterinary pathologist. Samples of the following tissues were fixed in 10% neutral buffered formalin, processed to glass slides in a standard manner and examined for abnormal tissue or gross lesions: adrenal glands, aorta arch, brain, bronchial lymph node, epididymes (if present), eyes with attached optic nerves, femur, gall bladder, gastro-intestinal tract (stomach, duodenum, jejunum, ileum, cecum, colon, rectum), entire heart, stifle joint with bone, kidneys (both), larynx and pharynx, liver, lungs, marrow smear, mesenteric lymph node, esophagus, ovary (if present), pancreas, pituitary gland, prostate (if present), sciatic nerve, skeletal muscle, skin and mammary gland, spinal cord, spleen, sternum, submandibular lymph node, submandibular salivary gland, testes (if present), thymus gland, thyroid and parathyroid glands, tongue, trachea, urinary bladder, and uterus, cervix, and vagina (if present). The histological evaluation was conducted by a certified veterinary pathologist. Prior to submersion in formalin, selected tissues were weighed (paired adrenal glands, entire brain, epididymis, heart, paired kidneys, liver, paired ovaries, pituitary gland, prostate, spleen, testis, thymus, paired thyroid and parathyroid glands, uterus, cervix, and vagina).

2.12 | Statistics

All data were analyzed with the statistical software package SAS® (version 9.2, SAS 9.2 Help and Documentation, SAS Institute Inc., 2002–2009, Cary, NC, USA). The following endpoints were analyzed: body weight, clinical pathology variables (hematology, coagulation, clinical chemistry, urinalysis), organ weights, food consumption, results of ophthalmoscopic and veterinary examinations, and ECG variables. Each treated group was analyzed compared to the control group.

Endpoints measured only once were analyzed using analysis of variance (ANOVA) with classification variables treatment, sex and their interaction, and block (random). Endpoints measured more than once post-treatment and at least once pretreatment were analyzed using repeated-measurements analysis of covariance (RMANCOVA), with the baseline value as covariate and the classification variables treatment, time, sex and their interactions, and block (random). The

TABLE 2 Frequency of observed clinical signs

	# of animals affected					# of incidence			
Variable/Group[a]	0	1×	2×	4×	*p* value[b]	0	1×	2×	4×
Gastro-intestinal tract									
Vomiting	0/8	5/8	0/8	6/8	**.0007**	0	9	0	17
Loose feces	2/8	1/8	1/8	1/8	.8706	2	2	1	1
Skin and appendages									
Dermatitis	4/8	1/8	0/8	2/8	.0937	6	3	0	4
Eye									
Conjunctivitis	0/8	1/8	0/8	2/8	.2565	0	1	0	6

Differences between groups were interpreted from *p* values shown in bold.
[a]Groups show multiples of the maximum daily therapeutic dosage of benazepril.HCl (1.0 mg/kg) and pimobendan (0.5 mg/kg) in Fortekor PLUS®.
[b]Cochran–Mantel–Haenszel (CMH) test for homogeneity.

baseline value was taken as the mean of the two pretreatment values on days -10 and -3, with the exception of food consumption where it was the mean of values on days -9 to -1.

Data were log-transformed if that improved the normality of distributions, which was tested with the Shapiro–Wilk test.

In the event of *p* values less than .1 for group, group × sex and/or group × day effects, the three test article groups were compared to the control group in post hoc analyses using the Least Significant Difference test (LSD) with $p < .05$ interpreted as significant.

The frequency of adverse events was compared between groups using the Cochran–Mantel–Haenszel (CMH) test for homogeneity followed by Fisher's Exact test for post hoc comparisons, with $p < .05$ interpreted as significant.

No adjustment of *p* values was made for multiple comparisons, in order not to inflate the type II error.

3 | RESULTS

The administered dosages of the test article were controlled and confirmed to be close to the planned nominal dosages.

3.1 | Body weight and food consumption

For body weight there was a highly significant ($p = .0001$) day effect, as some of the dogs were still growing during the study. However, there were no significant ($p > .1$) group, group × sex or group × day effects (data not shown). There was a significant effect of week ($p = .03$) on feed consumption, but no significant ($p > .1$) group, group × sex or group × week effects. It was concluded that the test article had no significant effect on body weight or food consumption.

3.2 | General health observations

There were no significant treatment-related adverse findings in general health observations. The frequency of vomiting was significantly different between groups ($p = .0007$), and in the post hoc comparisons was significantly higher in the 1× ($p = .0256$) and 4× ($p = .0070$) groups compared to the control, but not in the 2× group (Table 2). In the absence of a dose-dependent effect, a possible association between the test article and vomiting was concluded. The frequency of conjunctivitis was higher in the 1× and 4× groups compared to the control (Table 2). No association with the test article was concluded due to the lack the statistical significance and no dose-dependency. The frequency of loose stools and dermatitis was higher in the control group in comparison with the test article groups.

3.3 | Veterinary and neurological examinations

No clinically relevant abnormalities attributable to the test article were detected during scheduled veterinary and neurological examinations (data not shown).

3.4 | Ophthalmological examinations

Apart from the cases of conjunctivitis mentioned previously, there were no differences between control and test article groups in the frequency or severity of other abnormalities observed during the ophthalmological examinations (data not shown).

3.5 | Electrocardiogram (ECG) examinations

Statistical analysis of the ECG data revealed significant ($p < .05$) effects for group, group × day or group × sex for heart rate, PR interval, QRS amplitude, and QT interval (Table 3). For heart rate, the normality assumption was not achieved ($p < .05$). Post hoc analyses demonstrated increased heart rate and QRS amplitude, and decreased PR and QT intervals, in one or more of the test article groups compared to the control group at one or more time points (Table 3, Figures 1–4). For heart rate, differences between the test article groups and the control were only significant in the post hoc analyses at the day 88 and 116 time points (Figure 1), with some evidence of a dose-dependent effect

TABLE 3 Results of statistical analyses using RMANCOVA for electrocardiogram (ECG) variables

	p values from RMANCOVA							
Variable	**Baseline**	**Group**	**Sex**	**Day**	**Group × sex**	**Day × group**	***p* value normality**	***p* < .05 in post hoc tests**
Heart rate	.0001	.3330	.5658	.0001	.2889	**.0042**	.0279	D88 2, 3 & 4 > 1 D116 4 > 1
P-wave duration	NA	.3949	.3190	.4201	.3949	.4587	.0001	
P-wave amplitude	.0006	.5745	.8622	.0001	.6767	.8358	.5010	
PR interval	.0001	**.0495**	.7129	.0012	.9277	**.0008**	.1151	D116 2 < 1 D144 4 < 1 D172 3 & 4 < 1
QRS duration	.8814	.2470	.4167	.0610	.5232	.1557	.0001	
QRS amplitude	.0001	.6439	.4965	.0001	**.0523**	**.0001**	.0711	D172 4 > 1 Male 3 > 1
QT interval	.0002	**.0013**	.8858	.0299	.8671	**.0478**	.3271	D88 2, 3 & 4 < 1 D116 2 & 4 < 1 D144 2 & 4 < 1
T-wave amplitude	.0256	**.0514**	.5123	.0265	**.0558**	.1148	.4724	D116 4 < 1 Male 2 > 1

D, day; NA, Not available.
Heart rate and T-wave amplitude data were log-transformed. Data of the other variables were not transformed.
Differences between groups were interpreted from *p* values shown in bold ($p < .1$ for group, group × sex or day × group effects). There were no significant ($p > .1$) day × sex or day × group × sex interactions for any variable (data not shown).
For post hoc tests, $p < .05$ was interpreted as significant.

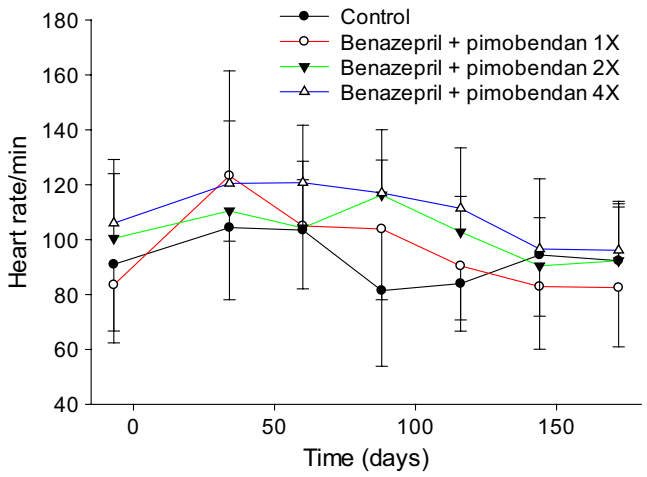

FIGURE 1 Heart rate measured by ECG. Data are mean and *SD*. The test article was administered each day on days 0 to 179

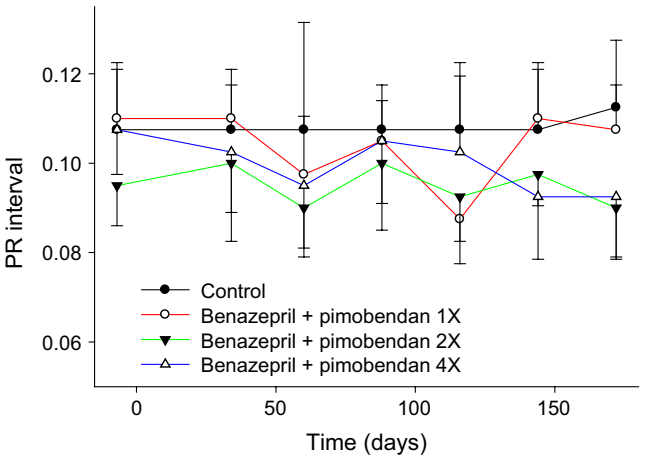

FIGURE 2 PR interval measured by ECG. Data are mean and *SD*. The test article was administered each day on days 0 to 179

of the test article in increasing heart rate. The mean (SD) heart rate during the 6-month treatment period in the control, 1×, 2×, and 4× groups was, respectively, 93.1 (18.0), 98.0 (21.1), 102.7 (12.7), and 110.8 (14.2) beats per min. Compared to the control, this represents 5.0%, 10.1%, and 18.8% higher mean values in the 1×, 2×, and 4× groups, respectively. The baseline heart rate differed between groups; however, mean (SD) change from baseline in heart rate in the control, 1×, 2×, and 4× groups was, respectively, 2.3 (21.0), 14.5 (17.7), 2.2 (14.4), and 4.8 (18.1.), respectively, that is, with no dose-dependency.

For the PR interval, and notably the QT interval, effects of the test article in decreasing the intervals were significant but there was only moderately strong evidence for dose-dependency (Table 3, Figures 2–3).

Although the group × day interaction was highly significant ($p = .0001$) for QRS amplitude, mean values were different between groups at baseline and there was little evidence of differences between groups for change from baseline, or for dose-dependent effects of the test article (Table 3, Figure 4). For T-wave amplitude, *p* values were <.1 but >.05 for group effect and group × sex interaction. However, there was no difference between groups for change from baseline and no dose-dependent effects of the test article (data not shown). It was concluded that there was insufficient evidence to conclude for a test article effect on either QRS or T-wave amplitude.

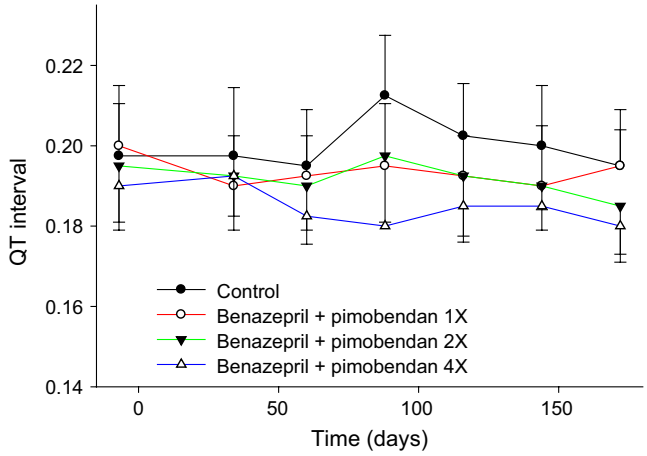

FIGURE 3 QT interval measured by ECG. Data are mean and *SD*. The test article was administered each day on days 0 to 179

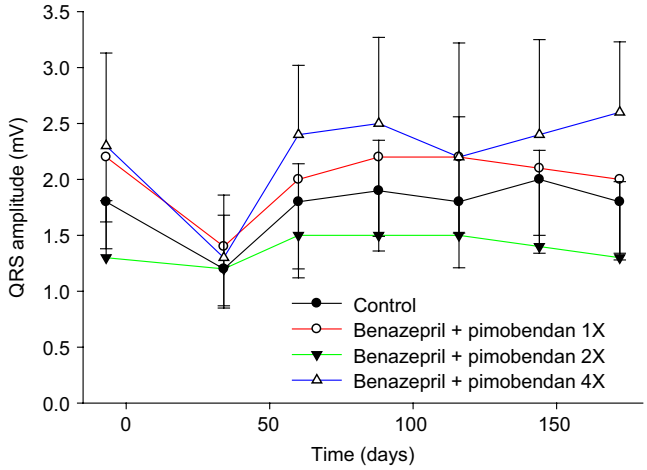

FIGURE 4 QRS amplitude measured by ECG. Data are mean and *SD*. The test article was administered each day on days 0 to 179

Any abnormality of the ECG was identified in seven dogs (87.5%) in all four groups, with no abnormality in one dog (12.5%). *p* values were therefore 1.0 for the overall and the post hoc between-group comparisons.

Abnormality of the ST segment structure of the ECG was identified in one dog (12.5%) in the control, 1×, and 2× groups, and in four dogs (50%) in the 4× group. Differences were not significant either for the all-group comparison ($p = .1765$) or the post hoc comparisons ($p = .2821$) of the 4× group to the control, 1×, or 2× groups.

3.6 | Clinical pathology

Results of statistical analyses are shown in Tables 4 and 5.

For the hematological variables, significant group, group × day, and/or group × sex effects at $p < .1$ were observed for 12 of the 15 variables (Table 4). Mean values for all variables remained inside the reference ranges and were not associated with any detected clinical effects. For all variables, significance in the post hoc tests was never observed in all treated groups or at all time points, and changes were not dose-dependent (data not shown). The findings were therefore considered not to be clinically relevant.

For coagulation variables, there were significant effects for group or its interaction with day and/or sex for activated partial thromboplastin time and fibrinogen (Table 4). The effects were not dose-dependent.

For the clinical chemistry variables, significant group, group × day, and/or group × sex effects at $p < .1$ were observed for 12 of the 22 variables (albumin, albumin/globulin ratio, alkaline phosphatase, amylase, chloride, cholesterol, creatine phosphokinase, gamma glutamyltranspeptidase, glucose, lactose dehydrogenase, phosphate, and total protein) (Table 5). Statistical analysis was not conducted for total bilirubin as most values were below the limit of quantification of the assay (1.7 μmol/L). In the post hoc analyses, there were statistically significant differences between one or more test article groups compared to the control group at one or more time points for amylase, chloride, cholesterol, glucose, phosphate, and total protein (data not shown). All amylase, chloride, cholesterol, glucose, and total protein values remained well within reference ranges or there was no clear dose-response, or both. The changes were therefore judged not to be biologically relevant. Decreases in plasma phosphate concentrations are associated with primary hyperparathyroidism in dogs, when they are accompanied by hypercalcemia. Although significant decreases in phosphate were observed, mean concentrations for all groups remained within the laboratory reference range at all times. Furthermore, there were no significant changes in plasma calcium and there was no evidence of hyperparathyroidism, as the parathyroid glands were classified as normal during both the necropsy and histological examinations. The changes in phosphate were therefore concluded not to be biologically relevant.

For the urinalysis, significant group × day and group × sex effects at $p < .1$ were observed for urine protein but not for urine specific gravity. The effects were not dose-dependent or clinically relevant.

All fecal samples were negative for occult blood. No abnormalities were detected during fecal examinations for color and consistency.

3.7 | Necropsy and histological evaluation

No treatment-related gross macroscopic changes were noted at necropsy (data not shown). All necropsy findings were assessed as spontaneously occurring background lesions of the nature commonly seen in this age, breed, and strain of dog.

For absolute organ weights, there was a significant ($p < .05$) sex effect for adrenal glands, heart and brain, but the group effect and group × sex interaction did not approach significance for any organ (Table 6). When the organ weights were corrected for body weight (relative organ weight = organ weight/kg), the sex effect persisted for adrenal glands ($p = .0003$) but not for heart ($p = .5259$) or brain ($p = .8088$). For organ weights corrected for body weight, there were no significant ($p > .1$) group or group × sex effects for any variable (data not shown). It was therefore concluded that there was no relevant effect of the test article on organ weights.

Histopathological evaluation did not identify any findings associated with the test article. Results from organs relevant to the clinical use of Fortekor PLUS® are shown in Table 7. There were no significant

TABLE 4 Results of statistical analyses using RMANCOVA for hematology and coagulation variables

Variable	*p* values from RMANCOVA								Transformation	*p* value normality
	Baseline	Group	Sex	Day	Group × sex	Day × group	Day × sex	Day × group × sex		
Basophil	.0001	.8046	.0001	.0001	.4722	**.0001**	.0001	**.0128**	Log	.9730
Eosinophil	.0001	**.0407**	.1375	.0001	**.0937**	**.0050**	.0080	.1176	Log	.3164
Hemoglobin	.0001	**.0071**	.7173	.0001	.4106	**.0001**	.0011	**.0003**		.0955
Hematocrit	.0001	**.0119**	.3836	.0001	.6061	**.0001**	.0038	**.0001**		.0500
Leukocyte	.0013	.2579	.0048	.0001	.1679	**.0489**	.0016	.0574	Log	.0260
Lymphocyte	.0001	.9458	.0279	.0001	.1284	**.0001**	.0589	**.0006**		.0001
MCH	.0001	.8827	.1072	.0001	.4644	**.0001**	.0012	.0537		.2586
MCHC	.0001	.9341	.0199	.0001	.6415	.1081	.1012	.0805		.1995
MCV	.0001	.1249	.2880	.0001	**.0930**	**.0001**	.0168	**.0034**		.0001
Monocyte	.0001	.4900	.1259	.0001	.3213	.2611	.0276	.4167	Log	.5655
Neutrophil	.0001	**.0733**	.0298	.0001	.4375	.2060	.0313	.1987	Log	.0011
Platelet	.0001	.7118	.5578	.0001	.8509	.4737	.8965	.2695		.0168
RBC	.0001	**.0395**	.1792	.0001	.4024	**.0001**	.0025	**.0047**		.0126
Reticulocyte	.0002	**.0001**	.5957	.0005	.9987	.1942	.3043	.2903		.0151
WBC	.0001	.4182	.0224	.0001	.1771	**.0129**	.0056	**.0001**	Log	.0001
APTT	.0001	**.0108**	.1481	.0001	.1812	.9039	**.0839**	.0881		.0001
Fibrinogen	.0288	.5439	.9017	.0001	.5081	**.0004**	**.0172**	.1755	Log	.5085
PT	.0001	.4161	.8289	.0001	.3705	.1191	.8243	.4109	Log	.0020

Differences between groups were interpreted from *p* values shown in bold ($p < .1$ for group, group × sex or day × group effects; $p < .05$ for day × group × sex). Data were either log-transformed (log) or not transformed.

APTT, activated partial thromboplastin time; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PT, prothrombin time; RBC, red blood cell count; WBC, total white cell count.

effects ($p < .05$) either for the all-group overall comparison (shown in Table 7) or for the post hoc comparisons of groups (data not shown).

A number of microscopic findings were observed with similar incidences in control and test article treated animals, and which are common incidental findings in dogs (Scudamore, 2012), for example, vacuolation of the adrenal cortex (zona glomerulosa and zona fasciculate).

Ectopic thyroid was recorded in the aorta of a female treated at 4× and in the heart of a female treated at 1×. Ectopic thyroid in the aorta and/or heart is a congenital finding occasionally observed in beagle dogs (Roth & Perentes, 2012) and has no toxicological relevance. Focal mural mineralization of the aorta was recorded in one male and one female treated at 2×, and two females treated at 1×. Mineralization of the root of the aorta is occasionally seen in young dogs as spontaneous background lesions and has no toxicological relevance (Scudamore, 2012). Cyst/cystic cleft was recorded in the pituitary gland and originates from remnants of embryonic ducts. Retinal changes such as pseudocyst in the eye are also congenital findings occasionally observed in beagle dogs. Both of these findings have no toxicological relevance. Ventricular dilation of the brain is occasionally seen in young beagle dogs as a spontaneous background lesion and has no toxicological relevance (Peckham, 2002). Various low-grade inflammatory lesions and/or lymphoid hyperplasia are common incidental findings in young beagle dogs and have no toxicological relevance (Chamanza et al., 2007; Peckham, 2002; Sato et al., 2012; Scudamore, 2012). Epithelial cyst formation is a congenital finding commonly observed in the thymus of beagle dogs and the cysts are thought to arise from brachial pouches (Scudamore, 2012). Hemosiderotic nodule in the spleen, tubular atrophy in the testes, cysts in the gall bladder, squamous metaplasia of the trachea, and decidualization of the uterus are all spontaneous lesions commonly seen in beagle dogs, and have no toxicological relevance.

There was therefore no evidence of any correlation between the histopathological changes and the test article, and all findings were interpreted as incidental changes.

4 | DISCUSSION

In this study, the administration of Fortekor PLUS®, a new tablet combination of benazepril and pimobendan, was well tolerated in adult healthy dogs at 1×, 2×, and 4× times the maximal recommended therapeutic dosage for 6 months.

As is standard in target animal safety studies, a large number of variables was evaluated and many were recorded at repeated time points. Results of the statistical analyses therefore need to be evaluated with caution, as many events of statistical significance at $p < .05$ and $p < .1$ would be expected as type I errors. As is standard in target

TABLE 5 Results of statistical analyses using RMANCOVA for clinical chemistry (and selected urinalysis) variables

Variable	*p* values from RMANCOVA								Transformation	*p* value normality
	Baseline	Group	Sex	Day	Group × sex	Day × group	Day × sex	Day × group × sex		
AG-R	.0001	.7375	.1443	.0001	**.0722**	.6230	.3010	.3556		.0812
ALP	.0001	.6756	.3186	.1098	**.0660**	**.0737**	.0808	**.0224**	Log	.0013
ALT	.0001	.3055	.5153	.3263	.1203	.4258	.1347	.4680	Log	.0001
AST	.0001	.1064	.8606	.0001	.1506	.8095	.1926	.3555	Log	.8870
Albumin	.0001	.1192	.0156	.0001	**.0224**	.8361	.4327	.9580		.0557
Amylase	.0001	**.0935**	.4990	.1703	.8047	**.0001**	.0037	.1557		.2483
Calcium	.0442	.5609	.2793	.0001	.6603	.3693	.8825	.5221		.0001
Chloride	.0102	.6193	.3013	.0001	.5254	**.0180**	.7362	.1639	Log	.0003
Cholesterol	.0001	**.0340**	.0001	.0014	.2536	**.0276**	.0974	.3868	Log	.0153
CPK	.0724	**.0014**	.2405	.0001	.3313	.6308	.0257	.1248	Log	.0001
Creatinine	.0001	.7152	.4676	.0001	.5245	.3336	.4732	.1596		.1658
GGT	.0001	.4246	.0240	.0001	**.0228**	.2940	.8504	.9769	Log	.0147
Globulin	.0001	.5909	.7451	.0106	.1338	.1926	.8607	.6028		.0610
Glucose	.0001	.8674	.7734	.0001	.5570	**.0051**	.7476	.8074	Log	.0922
LDH	.0001	**.0426**	.9901	.0001	.9580	.4369	.4291	.2935	Log	.0001
Magnesium	.0018	.3893	.0155	.0001	.5213	.2179	.6350	.1797	Log	.0001
Phosphate	.0001	**.0097**	.9727	.0001	.9216	**.0002**	.0977	.0703	Log	.0581
Potassium	.9132	.8465	.2605	.0012	.3148	.1595	.5314	.7594	Log	.0175
Sodium	.0302	.1436	.4336	.0001	.3323	.2668	.3041	.6220	Log	.0001
Total protein	.0001	.5669	.7291	.0685	.7109	**.0263**	.0740	.3306	Log	.0554
Urea	.0001	.3684	.5525	.0001	.9090	.6912	.0422	.4792	Log	.1266
Urine protein	.1164	.2747	.0011	.0007	.2101	**.0001**	**.0004**	**.0001**		.0001
Urine specific gravity	.0080	.3223	.4096	.0279	.3143	.7289	.8018	.2401		.3042

Differences between groups were interpreted from *p* values shown in bold ($p < .1$ for group, group × sex or day × group effects; $p < .05$ for day × group × sex). Data were either log-transformed (log) or not transformed.

AG-R, albumin/globulin ratio; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPK, creatine phosphokinase; GGT, gamma glutamyl transpeptidase; LDH, lactate dehydrogenase.

animal safety studies, a probable causal relation with the test article was therefore concluded by the combination of statistical significance plus evidence of a dose-related effect. To our knowledge, the dose-response relationships for benazepril and pimobendan have not been studied in combination, and therefore it is not known if interferences could occur.

No effects of the test article on general health, frequency of clinical signs or neurological or ophthalmological systems were detected. Although the frequency of vomiting and conjunctivitis was higher in the 1× and 4× groups compared to the control, they were not reported in the 2× group. Differences were statistically significant for vomiting but not for conjunctivitis. In the absence of dose-dependent effects, it was concluded that there was no relation with the test article for conjunctivitis but that an association was possible for vomiting. In contrast, the frequency of loose stools and dermatitis was higher in the control group in comparison with the test article groups. Vomiting was reported from target animal safety studies in healthy dogs with both benazepril (E. A. Kuntz & J. N. King, unpublished data) and pimobendan (US Freedom of Information Summary for Vetmedin®, 2007). In a field study in dogs with CHF, Fortekor PLUS® tablets were associated with significantly less vomiting compared to concomitant administration of benazepril (Fortekor® Flavor) and pimobendan (Vetmedin®) tablets (King et al., 2017). This result suggests that other factors in addition to the active ingredients contributed to the vomiting, for example the number of tablets. Reduced frequency of vomiting would be a clinically relevant benefit of this combination tablet.

There were significant effects of the test article in increasing heart rate, with associated reductions in PR and QT intervals, but values remained within acceptable limits. There was only moderately strong evidence that these observations were dose-dependent. These effects were most probably related to reflex tachycardia secondary to reduced systemic blood pressure, although this cannot be confirmed as blood pressure was not measured in this study. Both benazepril and pimobendan reduce blood pressure, mainly via systemic vasodilation (Boyle & Leech, 2012; Brands et al., 1993). The effect of the test article on heart rate in this study appeared to be biologically relevant as

TABLE 6 Summary of organ weights

Group[a]	0		1×		2×		4×		*p* values from ANOVA		
Organ weight (absolute [g])	Mean	*SD*	Mean	*SD*	Mean	*SD*	Mean	*SD*	Group	Sex	Group × sex
Thyroid & Parathyroid gland (right)	0.666	0.223	0.579	0.065	0.586	0.142	0.625	0.186	.7141	.7275	.9019
Thyroid & Parathyroid gland (left)	0.646	0.180	0.588	0.132	0.634	0.157	0.599	0.159	.8599	.6478	.9032
Spleen	75.037	22.891	68.800	39.187	81.550	21.175	78.488	22.966	.7199	.2220	.2946
Prostate gland	12.480	2.079	13.226	2.386	10.568	3.098	12.296	2.937	.3239	NA	NA
Liver with drained gall bladder	479.838	83.271	508.138	83.959	463.725	65.820	489.688	112.094	.7121	.5950	.1660
Adrenal glands	1.754	0.308	1.745	0.423	1.856	0.537	1.894	0.383	.8155	**.0058**	.6126
Kidney	73.863	20.749	76.088	15.731	73.425	8.376	77.613	12.254	.8945	.0587	.5673
Epididymis	5.531	0.835	5.302	0.502	5.535	1.216	5.932	1.169	.8066	NA	NA
Testis	16.675	3.271	19.375	1.826	18.200	3.672	19.650	2.237	.2026	NA	NA
Ovary	1.317	0.402	1.816	0.736	1.354	0.145	1.635	0.717	.4777	NA	NA
Uterus, Cervix and Vagina	25.125	15.331	28.900	17.203	17.150	5.844	27.850	26.435	.7662	NA	NA
Thymus gland	11.088	6.270	11.350	1.915	11.713	2.723	9.013	3.471	.5566	.9146	.5681
Heart	120.950	20.457	116.575	16.709	109.600	13.294	124.675	13.944	.1251	**.0019**	.8780
Brain	79.938	6.285	81.238	6.433	80.038	5.839	81.538	7.898	.9357	**.0469**	.3226
Pituitary gland	0.065	0.033	0.077	0.018	0.078	0.010	0.071	0.008	.4948	.3558	.7220

[a]Groups show multiples of the maximum daily therapeutic dosage of benazepril.HCl (1.0 mg/kg) and pimobendan (0.5 mg/kg) in Fortekor PLUS®.
Differences between groups were interpreted from *p* values shown in bold ($p < .1$ for group, group × sex).
NA, Not applicable.

mean heart rates were 5.0%, 10.1%, and 18.8% higher in the 1×, 2×, and 4× groups, respectively, compared to the control group. Modest reduction in the afterload is a desired outcome for the test article in clinical cases of CHF.

In previous studies, benazepril reduced systemic blood pressure in healthy dogs at dosages of 3–10 mg/kg, but not at the 1 mg/kg dosage used in this study (Ishibashi, Tatebe, Mitomi, Tanaka, & Imai, 1991). Benazepril has also been shown to reduce blood pressure in dogs with experimental heart failure (Brands et al., 1993) and chronic renal failure (Mishina & Watanabe, 2008). In models of induced mitral regurgitation, pimobendan decreased blood pressure but did not increase heart rate at 0.25 mg/kg BID (Kanno et al., 2007) and reduced left atrial pressure at 0.25 and 0.5 mg/kg BID with a greater effect at 0.5 mg/kg (Suzuki et al., 2011). In a model in which cardiac function in dogs was severely depressed by pentobarbital, pimobendan at dosages of 1–10 mg improved cardiac function in a dose-dependent manner but did not increase heart rate (Satoh, Satoh, Imagawa, & Taira, 1993). Therefore, it appears probable that the blood pressure lowering effects of benazepril and pimobendan in healthy beagles are additive when used in combination.

Increased QRS complex amplitude can be caused by (left ventricular) heart enlargement (Molloy, Akin, Devereux, & Kligfield, 1992). Although a significant group × day interaction was observed for the QRS complex amplitude, it was concluded that there was no evidence for a test article effect due to lack of differences between groups for change from baseline and the absence of dose-dependent effects. This conclusion is consistent with the lack of effect of the test article on heart weight or cardiac histology at postmortem.

For T-wave amplitude, *p* values were <.1 but >.05 for group effect and group × sex interaction. It was concluded, however, that there was no evidence for a test article effect since there was no difference between groups for change from baseline and no dose-dependent effects. No significant effects of the test article were observed on the other ECG parameters of P-wave duration and amplitude, QRS duration or frequency of abnormal ECGs.

There were statistically significant differences for a number of clinical pathology variables but these were either not sustained, or were of no toxicological relevance, because values did not fall outside the reference ranges. None of these changes were associated with any clinical signs in the treated animals.

There were no macroscopic or histopathology findings that could be attributed to administration of the test article in this study. Statistical analysis of the data revealed no significant findings for organ weights. A clinically relevant finding in this study is the lack of effect of the test article on heart weight or morphology. In previous target animal safety studies in healthy dogs, reduction in heart weight was observed with benazepril in one study at the highest dosage (100–150 mg/kg per day for 13 weeks) but not at other dosages (1, 10 and 30 mg/kg for

TABLE 7 Incidence of histopathologic observations from microscopic postmortem evaluation of selected organs

Group[a]	# Observations/group				
Variable/Organ	0	1×	2×	4×	*p* value
Aorta	(8)	(8)	(8)	(8)	
No abnormality detected	8	6	6	7	.4562
Mineralization, mural, focal	0	2	2	0	.2060
Ectopic thyroid	0	0	0	1	.3769
Duodenum	(8)	(8)	(8)	(8)	
No abnormality detected	5	7	7	8	.2120
Dilated glands	3	1	1	0	.2120
Heart	(7)	(8)	(8)	(8)	
No abnormality detected	6	6	8	8	.2579
Myocardial degeneration, focal	1	0	0	0	.3152
Arteritis/periarteritis, focal	0	1	0	0	.3961
Ectopic thyroid	0	1	0	0	.3961
Oil red O stain positive for fat					
Minimal	6	5	7	6	.6180
Mild	0	1	0	0	.3961
Total incidence	6	6	7	6	.8773
Oil red O stain negative for fat	1	2	1	1	.8875
Ileum	(8)	(8)	(8)	(8)	
No abnormality detected	7	8	8	8	.3769
Inflammation, submucosal, muscularis, chronic active, localized	1	0	0	0	.3769
Jejunum	(8)	(8)	(8)	(8)	
No abnormality detected	8	7	8	8	.3769
Inflammation, submucosal, focal	0	1	0	0	.3769
Kidney	(8)	(8)	(8)	(8)	
No abnormality detected	3	2	5	2	.3618
Inflammatory cell infiltration, cortical ± medullary	0	1	0	2	.2565
Inflammatory cell infiltration, pelvic	5	5	3	4	.7101
Oil red O stain positive for fat					
Minimal	3	0	1	3	.1765
Mild	3	7	5	4	.2092
Total incidence	6	7	6	7	.8446
Oil red O stain negative for fat	2	1	2	1	.8446
Liver	(8)	(8)	(8)	(8)	
No abnormality detected	8	7	8	8	.3769
Inflammatory cell foci	0	1	0	0	.3769
Oil red O stain negative for fat	8	8	8	8	
Lung	(8)	(8)	(8)	(8)	
No abnormality detected	4	2	4	3	.6997
Inflammation, interstitial, focal	0	0	0	1	.3769
Inflammatory cell foci	0	2	0	1	.2565
Inflammation, chronic, focal	4	5	3	3	.7101
Pleural fibrosis, localized	0	0	0	1	.3769
Hair/skin embolus, focal	0	1	1	0	.5452
Stomach	(8)	(8)	(8)	(8)	
No abnormality detected	7	8	7	4	.0606
Mineralization, mucosal, focal	1	0	1	0	.5452

[a]Groups show multiples of the maximum daily therapeutic dosage of benazepril.HCl (1.0 mg/kg) and pimobendan (0.5 mg/kg) in Fortekor PLUS®. Figures in brackets represent the number of animals from which this organ/tissue was examined microscopically.
There were no significant effects ($p < .05$) either for the all-group overall comparison (shown in Table) or for the post hoc comparisons of groups (not shown).

13 weeks) or in a second study (15, 50 and 150 mg/kg for 12 months) (E. A. Kuntz & J. N. King, unpublished data). It is concluded that benazepril should have no biologically relevant effect on heart weight if administered without pimobendan at the dosages tested in this study (up to 4 mg/kg per day).

Effects of pimobendan on the heart have been reported from two target animal safety studies in healthy dogs. First, intravenous administration of 0.5, 2 and 8 mg/kg pimobendan once daily for 4 weeks resulted in dose-dependent increases in heart rate, mitral valve myxomatous thickening, left ventricular outflow tract endocardial thickening and ventricular muscle ischemic lesions (Schneider et al., 1997; US Freedom of Information Summary for Vetmedin® (2007)). Second, at 3

and 5 mg/kg orally for 180 days, pimobendan caused severe left ventricular hypertrophy with multifocal subendocardial ischemic lesions, myxomatous thickening of the mitral valves, mitral valve insufficiency murmurs, left atrial jet lesions, endocardial thickening of the left ventricular outflow tract, a granulomatous lesion within the right atrial myocardium, decreased blood pressure, increased heart rate, and a small increase in ventricular premature contractions (US Freedom of Information Summary for Vetmedin® (2007)).

In addition, effects of pimobendan on the heart have been reported from dogs with natural heart disease in two studies. First, myocardial hypertrophy was reported in two clinical cases after treatment with pimobendan for 10 months (0.33 mg/kg BID) or 5 months (0.29 mg/kg BID). The hypertrophy was partially reversed in one dog after substitution of pimobendan with benazepril (Tissier et al., 2005). Second, pimobendan (0.25 mg/kg BID) for 512 days increased the frequency of acute focal hemorrhages, endothelial papillary hyperplasia and infiltration with glycosaminoglycans of the chordae tendineae of the mitral valves (Chetboul et al., 2007).

In spite of these reports of adverse effects of pimobendan on the canine heart in some studies, well-controlled clinical trials have demonstrated that pimobendan has a favorable benefit to risk profile in dogs with CHF (Boswood et al., 2016; Häggström et al., 2008).

The finding from this study that the combination of benazepril and pimobendan in Fortekor PLUS® had no detected effect on the heart or its valves in healthy dogs might be related to one or both of the following: first, that the 2 and 4× multiples of the dosage of oral pimobendan in our study (up to 2.0 mg/kg per day) were too low to cause cardiac pathology, as the objective of the study was to set a margin of safety; and second, the possibility that benazepril might have reduced the effects of pimobendan in causing myocardial hypertrophy and mitral valve lesions. Testing this last hypothesis would require an additional parallel-group design comparing benazepril alone, pimobendan alone and their combination. It is well established that ACEIs reduce ventricular hypertrophy in humans (Pitt, 1998).

The principal limitations of the study are listed below.

First, the ECG recordings were performed in conscious and lightly restrained dogs and analyses were performed over a time period of 30 s. This is a standard approach for target animal safety studies. Our results therefore do not preclude the possibility of the test article causing uncommon disturbances to the cardiac rhythm.

Second, the study animals were healthy beagle dogs. The results therefore cannot be simply generalized to the target population for Fortekor PLUS®, that is, dogs of various breeds with CHF. In a field study in dogs with CHF conducted in Japan, Fortekor PLUS® tablets had noninferior efficacy, were well tolerated and were associated with significant less vomiting compared to administration of Fortekor® and Vetmedin® tablets separately (King et al., 2017).

5 | CONCLUSIONS

Fortekor PLUS® tablets were well tolerated in adult healthy dogs over an extended period of 6 months when administered orally BID at dosages up to four times the maximum recommended therapeutic dosage (1.0 mg/kg benazepril.HCl per day and 0.5 mg/kg pimobendan per day). Treatment-related effects included increased heart rate and occasional vomiting. No effects on heart weight or cardiac morphology were detected.

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COMPETING INTERESTS

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CONTRIBUTIONS

The study was designed by EK. The statistical analyses were conducted by GS and the results interpreted by EK, JG and JNK. The manuscript was drafted by EK and JNK. All authors contributed to and approved the final manuscript.

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