Supplement 2

Complete Materials and Methods

Study Design

This was a prospective, multicenter, double-blind, randomized, placebo-controlled, non-pivotal (exploratory) field study. The study protocol was prepared by the study sponsor in collaboration with independent cardiologists (JR, KS, and VLF) and approved by an ethical review committee at each site where this was required.

Cats

Client-owned cats with prior (within the past 60 days) CHF secondary to HCM but without signs of congestion and heart failure at the time of enrollment were recruited by board-certified cardiologists at 10 centers (6 in the United States and 4 in Europe) between 2011 and 2013. Because of the non-pivotal (exploratory) nature of the study and absence of any published data in cats with HCM using a dose increase of furosemide as a component of the primary endpoint, and considering feasibility (anticipated length of enrollment period <18 months), a sample size of approximately 40 cats per treatment group was chosen. All clients of cats enrolled gave written informed consent for participation.

Inclusion criteria. Cats with body weight ≥ 2 kg, aged ≥ 12 months, with HCM and recent (but not current) diagnosis of CHF, clinical euvolemia, and hematocrit and total plasma protein concentration within the laboratory reference range were eligible. Presence of HCM was confirmed by a board-certified cardiologist in cats with increased left ventricular (LV) end-

diastolic wall thickness ≥ 6 mm of unknown cause as determined by transthoracic two-dimensional (2D) or M-mode echocardiography.[1,27] Cats with and without dynamic LVOTO were included. Obstructive HCM was defined by the presence of dynamic LVOTO with a peak systolic pressure gradient (PG) across the obstruction ≥ 30 mmHg as assessed by continuous wave Doppler and a late-peaking Doppler flow profile[21,22,28] while avoiding contamination of the outflow signal with mitral regurgitation. Non-obstructive HCM was defined by a peak systolic PG across the LVOT <30 mmHg. Cats had to be clinically asymptomatic at enrollment without evidence of pulmonary edema and pleural effusion but with a history of clinical and radiographic evidence of CHF within the last 2 months (≤ 60 days). One of the following diagnostic criteria had to be met for a cat to be considered to have CHF: 1) medical record documentation of thoracic radiographs from the investigator's site to support the diagnosis of CHF (cardiogenic pulmonary edema or pleural effusion or both), 2) historical diagnosis of CHF made by a board-certified cardiologist, 3) CHF based on thoracic radiographs provided by referring veterinarians and confirmed by the investigator, and 4) in situations where radiography could not be performed before treatment because of instability of the cat with clinical evidence of tachypnea, open mouth or labored breathing, response to treatment with furosemide, thoracocentesis, or some combination of these. After stabilization evidence of HCM on echocardiography severe enough to be compatible with prior CHF was a general requirement for enrollment. Center was not used as a stratification factor for enrollment.

Exclusion criteria. Conditions other than HCM capable of causing LV wall thickening; cardiac arrhythmias judged clinically relevant at the discretion of the investigator such as ventricular and supraventricular tachycardia, 3rd degree AV block, and atrial fibrillation; cases of CHF precipitated by known non-cardiac events such as parenteral fluid administration, treatment with

depot corticosteroids, anesthesia, and prior surgery; concurrent pulmonary or bronchial disease and heartworm infection; arterial thromboembolism; presence of intracardiac thrombi; systemic hypertension (repeated systolic blood pressure ≥ 160 mmHg); endocrinopathies; and, moderate to severe azotemia (BUN > 60 mg/dL [>21 mmol/L] and Creatinine > 2.5 mg/dL [>221 μmol/L]) were reasons leading to exclusion. Cats receiving ≥ 1 of the below treatments were not enrolled: sedation with ketamine, dexmedetomidine; treatment within the past 12 hours with nitroglycerin; treatment within the past 24 hours with angiotensin converting enzyme (ACE) inhibitors, antiarrhythmic drugs, diuretics other than furosemide, and antiplatelet medications other than clopidogrel; and treatment with anticoagulants, pimobendan within the past 7 days, and beta receptor blockers within the past 10 days.

Post-inclusion removal criteria included withdrawal of owner consent, development or worsening of dynamic LVOTO 2-5 hours post-medication after the first dose on Day [D] 0; increase in systolic LVOT PG of > 25 mmHg in a previously obstructive cat or development of a systolic LVOT PG > 50 mmHg in a previously non-obstructive cat), total daily furosemide dose > 10 mg/kg; development of adverse events necessitating unblinding; concomitant cardiovascular medications deemed necessary by the investigator; arterial thromboembolism; removal deemed necessary by the investigator for animal welfare reasons; and, discovery post-enrollment that the animal did not meet inclusion criteria.

Randomization and Allocation

Each cat that deemed eligible during screening examination (on Day -1 [D-1] or D0) was stratified by the presence or absence of dynamic LVOTO and subsequently randomized within their respective stratum to receive either pimobendan or placebo in a 1:1 allocation ratio.

Blinding

Investigators, owners, study monitors, and statisticians were blinded to treatment allocation during the study period. Access to the blinding code for the study group was limited to individuals who were otherwise independent of the study. In the event of a medical emergency; or if the study endpoint was reached; or at the particular request by the cat owner; predefined procedures were available to permit immediate disclosure of the study medication. In the event of premature unblinding the cat would be censored from the study at that time.

The study medication (placebo and pimobendan) was supplied as visually indistinguishable tablets. Sufficient tablets for treatment until the next visit were supplied to the owner on D0 and on each scheduled re-examination day until the study was completed. The tablet was offered to the cat for voluntary ingestion or administered directly into the mouth and was given at the same time the other medications were administered. Treatment started on D0.

Study Medication

Pimobendan tablets (Vetmedin® Flavour tablets 1.25 mg in the Europe, Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany and Vetmedin® Chewable tablets 1.25 mg in the United States, Boehringer Ingelheim Vetmedica Inc., Saint Joseph, MO) and placebo were identical in terms of appearance, smell, and taste at all study sites. Both pimobendan and placebo were administered orally twice daily at the same dose, depending on body weight: 2 to 3.1 kg (0.5 tablet), >3.1 to 5.2 kg (1 tablet), >5.2 to 7.2 kg (1.5 tablets), and > 7.2 kg (2 tablets). The total target dose of pimobendan was 0.6 mg/kg/d, divided into 2 equal portions administered approximately 12 hours apart. The dose of the study medication was not adjusted throughout the study.

Concomitant Treatments

Concomitant administration of furosemide (Furozenol® 10 mg tablets in Europe and Salix® 12.5 mg tablets in the United States) and clopidogrel (Plavix® 75 mg tablets) was allowed. The necessity and dose of these treatments were at the discretion of the individual investigators. After the primary endpoint was reached or the study was completed, the study drug was discontinued, and cats received additional treatments and adjusted doses of current treatments as deemed medically indicated at the investigator's discretion.

Population analyzed

For the purpose of statistical analysis, 4 cat populations were defined as follows: the safety set (SAF), the full analysis set (FAS), the per-protocol set 1 (PPS1), and the per-protocol set 2 (PPS2; Fig. 1). The SAF set consisted of all cats that were randomized and received at least 1 dose of the study medication. The FAS set was a subset of SAF with any cats violating inclusion criteria removed. The PPS1 population consisted of all cats of the FAS that reasonably complied with the protocol. Minor deviations from the ideal still may have occurred, but major protocol deviations affecting ability to assess treatment success led to exclusion from this protocol set. Finally, the PPS2 population consisted of all cats of the PPS1 population, but with removal of cats that fulfilled the post-inclusion withdrawal criterion 'development or worsening of dynamic LVOTO on D0'.

Schedule of Events

Before enrollment, all cats underwent a physical examination, blood pressure measurement, a complete blood cell count, and blood biochemical analysis including total plasma thyroxine concentration, thoracic radiography, ECG, and echocardiography (Table S1). Whenever possible,

examinations were performed with the cats non-sedated. If sedation was used, the same type and dose had to be used for repeated examinations. Cats were re-examined on D10, D60, and D180, and updates from the owners via telephone interviews were obtained on D90, D120, and D150. Unscheduled visits, if applicable, were made if a cat was showing clinical signs, if owner concerns were expressed, or when cats reached a study end point. An owner dosing log was conducted daily throughout the study.

Endpoints

The primary endpoint of the study was successful outcome at D180, defined as remaining in the study without an increase in furosemide dose. Withdrawal from the study for any reason before D180, or an increase in furosemide dose therefore would indicate failure to meet the primary endpoint. The secondary endpoints were time to withdrawal from the study; time to morbidity or mortality; time to first furosemide escalation; time to furosemide dose > 10 mg/kg/d; time to hospitalization for CHF; time to requiring precluded medications; time to aortic thromboembolism (ATE); and increase of severity of LVOTO (as defined previously). Decisions regarding dose escalation of furosemide (due to recurrence of signs of CHF using criteria prospectively determined) and initiation of precluded medications were made at the discretion of the individual investigator.[3]

Diagnostic Methods

The sequence of applied study methods is summarized in Table S1. Cardiac auscultation was performed and heart rate, rhythm, presence of a gallop sound, and presence and intensity of a heart murmur were recorded. Rate of respiration was determined. Body weight was measured at each visit. Doppler blood pressure measurements,[29] thoracic radiography,[30,31] transthoracic

echocardiography (exclusively performed by the investigators),[32] and electrocardiography[33] were performed and analyzed as previously reported (Supplement 1).

Safety Monitoring

Safety was monitored throughout the study, and nature, frequency, and outcome of adverse events in the SAF population (n=82) were recorded. Adverse events were classified following the principles of Good Veterinary Practice on the basis of System Organ Class. For general safety aspects, the systolic PG across the LVOT was monitored by repeating echocardiographic measurements 2 to 5 hours post medication (the expected time of peak positive inotropic effect of pimobendan based on available data in dogs at the beginning of the study) on D0, D10, D60, and D180 and during any unscheduled visit. Development of new LVOTO or increased severity of existing LVOTO at baseline under the same diagnostic conditions raised safety concerns and were addressed under the premises outlined below. During the study, an independent, unblinded pharmacovigilance scientist of the study sponsor familiar with the protocol and the disease but in no way associated with the study monitored the data pertaining the systolic PG across the LVOT and clinical signs 2 to 5 hours post administration of the drug on D0. A Data and Safety Monitoring Board was set up that included study sponsor personnel and independent cardiology consultants blinded to the study site and specific cats enrolled (JR and KES). This Data and Safety Monitoring Board would be responsible for critical review of the study if the following *a-priori* criteria were met: development of LVOTO (defined as systolic PG > 50 mmHg) in previously non-obstructive cats; worsening of LVOTO in previously obstructive cats (defined as an increase systolic PG > 25 mmHg) in at least 3 of the first 10 cats treated with pimobendan; or if 3 consecutive cats treated with pimobendan had new development or worsening of pre-existing LVOTO on D0, as defined above.

Statistical Analysis

Statistical analysis was performed using commercially available software (SAS® System Version 9.2, SAS Institute, Cary, NC). Normality of data was assessed by visual inspection, the Kolmogorov-Smirnov test, and for some variables the D'Agostino & Pearson test. The null hypothesis "success rate of cats treated with pimobendan is equal to success rate of cats treated with placebo" was tested against its alternative hypothesis by means of a 2-sided Cochran-Mantel-Haenszel test[38,39] controlled for LVOTO as stratification variable. Adjusted Mantel-Haenszel type odds ratios (OR) with 95% confidence intervals (CI) were provided to quantify the treatment effect. The Breslow-Day test[40] was used to assess homogeneity among LVOTO subgroups. To evaluate the sensitivity of the primary analysis, logistic regression analysis was performed with treatment and LVOTO as fixed effects and the continuous covariate furosemide dose (mg/kg) as baseline. Additional sensitivity analysis was done using a logistic regression model encompassing 'center' as a random effect. The 'center' effect also was evaluated visually by graphical display considering individual centers. Group differences for time-to-event secondary endpoints were evaluated using proportional hazard regression analysis with main effects of treatment and LVOTO. Cats that had experienced no events were censored on D180 or at the day of study removal. Effects of treatment, LVOTO, sex, age, body weight, diastolic class, and furosemide dose on D0 on outcome were analyzed using various logistic regression models. A P value $\leq .05$ and ORs with 95% CIs not including 1.0 were considered significant.

References

1. Luis Fuentes V, Abbott J, Chetboul V, et al. ACVIM consensus statement guidelines for the classification, diagnosis, and management of cardiomyopathies in cats. *J Vet Intern Med* 2020;34:1062-1077.

3. Ferasin L, DeFrancesco T. Management of acute heart failure in cats. *J Vet Cardiol* 2015:17:S173-S189.

21. Gersh BJ, Maron BJ, Bonow RO, et al. 2011 ACCF/AHA guideline for the diagnosis and treatment of hypertrophic cardiomyopathy. A report of the American College of Cardiology Foundation/American heart Association task Force on practice guidelines. *Circulation* 2011;124:e783-e831.

22. Elliott PM, Anastasakis A, Borger MA, et al. 2014 ESC guidelines on diagnosis and management of hypertrophic cardiomyopathy. *Eur Heart J* 2014;35:2733-2779.

27. Fox PR, Liu SK, Maron BJ. Echocardiographic assessment of spontaneously occurring feline hypertrophic cardiomyopathy. An animal model of human disease. *Circulation* 1995;92:2645-2651.

28. Coleman AE, DeFrancesco TC, Griffiths EH, et al. Atenolol in cats with subclinical hypertrophic cardiomyopathy: a double-blind, placebo-controlled, randomized clinical trial of effect on quality of life, activity, and cardiac biomarkers. *J Vet Cardiol* 2020;30:77-91.

29. Acierno MJ, Brown S, Coleman AE, et al. ACVIM consensus statement: Guidelines for the identification, evaluation, and management of systemic hypertension in dogs and cats. *J Vet Intern Med* 2018;32:1803-1822.

30. Guglielmini C, Diana A. Thoracic radiography in the cat: identification of cardiomegaly and congestive heart failure. *J Vet Cardiol* 2015;17:S87-S101.

31. Litster AL, Buchanan JW. Vertebral scale system to measure heart size in radiographs of cats. *J Am Vet Med Assoc 2000*;2016:210-214.

32. Schober KE, Chetboul V. Echocardiographic evaluation of left ventricular diastolic function in cats: hemodynamic determinants and pattern recognition. *J Vet Cardiol*. 2015;17:S102-S133.

33. Tilley LP. *Essentials of Canine and Feline Electrocardiography.* 4rd edition, Philadelphia, PA: Lea & Febiger; 1993.

38. Cochran WG. Some methods for strengthening the common $\chi 2$ tests. *Biometrics* 1954;10:417-451.

39. Mantel N, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. *J Natl Cancer Institute* 1959;22:719-748.

40. Breslow NE, Day NE, Halvorsen KT, et al. Estimation of multiple relative risk functions in matched case-control studies. *Am J Epidemiol* 1978;108:299-307.