

INFLUENCE of ABCB1 GENOTYPE in COLLIES on the PHARMACOKINETICS and
PHARMACODYNAMICS of LOPERAMIDE in a DOSE-ESCALATION STUDY

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Role of ABCB1 Genotype on Drug PKs and Pharmacodynamics in Collies

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Non Standard Abbreviations: wt, wild-type; Mut; homozygous mutant; Fg, fraction of drug absorbed from the intestine; 3D, Collies that only receive the three lowest doses of loperamide; 4D, collies that received all four doses of loperamide; HNS, heterozygous mutant-ivermectin nonsensitive; HS, heterozygous mutant-ivermectin sensitive; IVMNS, ivermectin-nonsensitive; IVMS, ivermectin sensitive

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ABSTRACT

Thirty three Collies (14 male and 19 female) were used in a dose-escalation study to determine the impact of ABCB1 genotype on loperamide pharmacokinetics (PK) and pharmacodynamics (PD). Loperamide was orally administered in four ascending doses (0.01, 0.05, 0.1, or 0.2 mg/kg) over a four week period to fasted Collies. Comparisons were made within each dose to genotype, phenotype, and whether Collies received 3 (3D) or 4 (4D) loperamide doses. The 3D and 4D groupings had statistically differences in systemic drug exposure (defined by the area under the concentration vs time profile estimated from time zero to the last quantifiable drug concentration, AUC_{0-last}). In contrast, statistical differences in AUC_{0-last} only occurred in the comparison between wild-type (WT) Collies versus homozygous mutant (Mut) Collies administered 0.1 mg/kg. Statistical differences in the proportionality relationship were observed when comparing 3D to 4D Collies, and the WT to Mut Collies. Intersubject variability in drug exposure tended to be twice as high between Mut and WT Collies. Associations were observed between systemic drug exposure and ataxia or depression, but not between systemic drug exposure and mydriasis or salivation. Thus, Collies expressing the greatest sensitivity to CNS-associated effects of loperamide (Mut) tended to have higher drug exposure compared to those less sensitive to the adverse effects of loperamide. Genotype and phenotype only partially explained differences in loperamide PK and PD, suggesting this relationship may not be straightforward and that other factors need to be considered. Accordingly, the PD and PK of one P-gp substrate only partially predicted the likelihood of adverse responses to unrelated substrates.

INTRODUCTION

The ABCB1 gene encodes for the efflux transporter, P-glycoprotein (P-gp). P-gp is a major efflux pump for a wide variety of human and veterinary drugs (Sadeque et al., 2000; Elkiwere et al., 2009).

Collies possessing a 4 nucleotide deletion (the ABCB1-1 Δ genotype) results in a frame shift mutation that yields a truncated, non-functional P-gp molecule (Mealey et al., 2001). Collies homozygous for this genotype (Mut) have an increased risk of having toxic responses to P-gp substrates as compared to those seen in the wild-type (WT) counterpart (Neff et al., 2004; Geyer et al., 2005; Hugnet et al., 2005; Mealey et al., 2005; Geyer et al., 2007; Gramer et al., 2010).

Examples of drugs reported to have canine genotype-associated toxicities include ivermectin (IVM), milbemycin, moxidectin, digoxin, mexiletine, loperamide and vincristine (Tanquilli et al., 1991; Staley & Staley, 1994; Hugnet et al., 1996; Sartor et al., 2004; Henik et al., 2006; Mealey et al 2008). The response of heterozygous Collies to these various compounds is not fully understood.

In humans possessing the 2677TT & 3435TT-associated P-gp polymorphism, both sedation and elevated plasma loperamide concentrations were observed at pharmacologically relevant doses (Skarke et al 2003). However, conflicting reports have been published on the impact of this ABCB1-1 Δ polymorphism on canine plasma loperamide concentrations. For example, Kitamura et al., (2008) observed that a pharmacological dose of loperamide (0.01 mg/kg) was associated with elevated plasma loperamide levels in Collies homozygous for the ABCB1-1 Δ mutation.

Conversely, no corresponding differences in loperamide PK were reported at a suprapharmacological dose of 0.2 mg/kg (Mealey et al., 2010). While it has been suggested that this discrepancy may reflect a dose-associated saturation of intestinal P-gp activity (Dufek et al., 2013), confirmation of the underlying cause for this difference in observations remains undetermined. Accordingly, further evaluation of this question is warranted.

To explore this issue, a dose escalation study was conducted to determine if P-gp saturation could be responsible for the lack of effect on loperamide pharmacokinetics (PK) as observed by Mealey. Furthermore, we matched these PK responses to clinical toxic outcomes to determine if any PK/pharmacodynamic (PD) correlations could be identified. Lastly, the questions remained as to whether dogs heterozygous for this defect behaved in a manner similar to the WT Collies or Mut Collies and if prior adverse reactions to IVM are predictive of loperamide toxicity.

MATERIALS & METHOD

Animals. Thirty-three intact Collies (14 males and 19 females) were enrolled in this study; all Collies were research animals obtained from a research colony, and returned at the end of the study. The ABCB1 genotype and sensitivity to IVM (0.1 mg/kg) of each Collie had been determined by the research colony prior to enrollment. All homozygous mutant Collies were sensitive to IVM. The Collies were individually housed and were kept in a facility in which the temperature was maintained between 68-72°F. There was a three week acclimation period prior to study initiation. The Collies were fed once daily (Pride 22/16; The Hyland Co, KY); this was

the identical feed offered to the Collies at the research colony. Water was provided *ad libitum* using nipple waterers and individual bowls of water. The Collies were provided at least once daily the ability to socialize and exercise with other Collies. Each Collie was also provided various forms of enrichment. All animal activities were approved by the FDA's Center for Veterinary Medicine Institutional Animal Care and Use Committee. The average age, weight, and gender composition are listed in Table 1. The Table also lists the numbers of Collies in each group that received just the three lowest doses of loperamide, or received all four doses (see below also).

Dose Groups: The Collies were arranged into four dosing groups based on genotype and sensitivity to CNS toxicities induced by IVM. The four groups (and number per group) were wild-type (7), heterozygous mutant, non-IVM sensitive (9), heterozygous mutant-IVM sensitive (7), and homozygous mutant (10). Collies were grouped according to their genotype of WT, Mut or heterozygous. The heterozygotes were further categorized into IVM-sensitive (HS) or IVM non-sensitive (HNS), for a total of 4 treatment groups. As genotypic/phenotypic information was known prior to study enrollment, the Collies were randomly ordered within a given study group. This ranking served as the basis for dosing group assignment. Prior to dosing group assignment, all Collies were assigned a random number using a random number table for purposes of clinical observations to ensure those making clinical observations were masked to genotype/phenotype.

Loperamide Administration. All Collies were weighed prior to initiation of the dose-escalation study. An over-the counter generic loperamide solution (1 mg/5 mL) was administered using an oral dosing syringe. The same product lot was used throughout the study.

Collies scheduled to receive loperamide were fasted overnight, and fed approximately 10 hr after loperamide administration. Dosing of Collies within each group occurred in 5 minute intervals. Loperamide was administered at doses of 0.01 mg/kg, 0.05 mg/kg, 0.10 mg/kg and 0.20 mg/kg, starting at the lowest dose. All Collies were administered one dose per week, with a 7 day wash-out separating administration of loperamide dose.

Plasma Collection and Observation for Clinical Signs of Toxicity.

All Collies had an indwelling catheter placed into the cephalic vein immediately prior to loperamide administration. The catheter was secured in place using surgical tape and Vetwrap™, with an injection cap sealing the end of the catheter. The catheters remained in place through the entire blood sampling period and were removed after the 24 hr sample collection. Catheter patency between sampling was maintained with heparinized saline (10 U/mL). Blood samples (approx. 5 mL per collection period) were collected at 0 hr (prior to loperamide administration) and at 1, 2, 5, 10 and 24 hr after oral administration of loperamide. Prior to the 0 hr sample collection, the dogs were observed for clinical signs of central nervous system (CNS) toxicity (depression, ataxia, mydriasis, &/or salivation). The individuals evaluating each Collie were masked to treatment, genotype, and phenotype.

A pre-defined 4 level scoring system for depression, ataxia, mydriasis, and salivation was employed to assess the clinical impact of loperamide administration (Fassler et al., 1991; Paul et al., 2000). The scoring criteria for each parameter ranged from normal (0) through severe (3), with narrative descriptions included for each scoring category and parameter. A single standardized scoring sheet was used for each Collie at each observation time. These observation

sheets were collected by the study director at the end of each observation period to prevent observer bias during subsequent observation periods. Once a Collie exhibited any signs of CNS toxicity, loperamide administration was discontinued and the dog did not receive higher doses of loperamide. While excessive, life-threatening adverse events were not anticipated, naloxone and other supportive therapies were available if needed.

Loperamide Analysis

The method, slightly modified from a published procedure (Ganssmann et al., 2001), is comprised of sample extraction and instrumental analysis. First, 0.5 mL internal standard solution (D, L-methadone, 1 ng/mL in borate buffer, pH 8.5) was added to an aliquot of 0.5 mL plasma sample, followed by addition of 1 mL ethyl acetate for extraction. After centrifugation, 700 μ L of the supernatant was evaporated to dryness, and the residue was reconstituted with 50 μ L of mobile phase (4 mM ammonium acetate buffer + acetonitrile + methanol, 14:13:13 v/v/v). A Shimadzu Prominence system, comprised of two LC20AD pumps, a DGU20A5 degasser, a SIL-20AC auto-sampler, and a CTO-20A column oven, was used for liquid chromatograph, with a Zorbax Eclipse XDB-C8 column (150 x 2.1 mm, 5 μ m). Mobile phase was run in isocratic mode at 0.25 mL/min for 5 minutes, while the column oven temperature was kept at 30°C. For signal detection, an AB Sciex 4000 Q-Trap mass spectrometer equipped with TurboIonSpray source (run in positive mode) was used. The ionization source was set at 550°C, 5000 V, and optimized interface gas supply rates. Two transitions were monitored for loperamide (“Lop1”, 477.3 \rightarrow 266.1 m/z, and “Lop2”, 477.3 \rightarrow 210.1 m/z), and one transition for desmethylloperamide (DML, 463.3 \rightarrow 252.1 m/z), and methadone (310.2 \rightarrow 265.1 m/z) respectively.

The sum of the 2 transitions (Lop1+Lop2) was used for loperamide quantitation, and a matrix-matched standard curve (with methadone as internal standard) was constructed for each batch. Retention time for loperamide was around 3.3 min, while DML eluted about 0.6 min earlier. Lacking reference standards for DML, estimates of their respective concentration were made based on the calibration curve of the Lop1 transition (Loperamide) with internal standard correction. In addition, the ratio of Lop1/Lop2 was used to verify identity of Loperamide in positive samples, along with retention time.

The method was validated over a linear quantitation range of 0.01 ng/mL to 10 ng/mL (after correction with concentration factor) for loperamide in canine plasma, at four spiking levels (5, 0.5, 0.05, and 0.01 ng/mL respectively), in multiple days. Linear regression with r^2 higher than 0.99 was achieved in all batches. The average recoveries at these levels were between 98% and 104%, with inter-day precision (described as relative standard deviation) ranging from 5% to 15%. No significant interference or carryover was found in blank control samples. An estimate of detection limit was made based on the instrument response in blank controls (0.0023 ng/mL), which was well below the lower limit of quantitation. For verification of analyte identity, all positive controls (N=19) met pre-set confirmation criteria, as none of the five blank controls did. Loperamide was also found to be stable in extract (refrigeration) for at least 24 h, and in plasma (<-70°C) over the period of analysis. Lastly, performance of the method was retrospectively reviewed with the spiked pre-dosing plasma samples from different collies that were analyzed in various days. The average accuracy was 104.6% (N=111) with an 11.6% relative standard deviation.

PK Analyses

The data were evaluated on the basis of Collie genotype/phenotype groupings, whether Collies previously exhibited IVM sensitivity, and if the Collies received all four doses of loperamide (4D) or only received the three lowest doses of loperamide (3D) due to manifestation of CNS toxicity at one of the lowest three doses of loperamide.

A noncompartmental model approach using the observed total loperamide concentrations (free plus bound to plasma proteins) were evaluated on the basis of the area under the concentration/time profile (AUC), and terminal elimination half-life (Thalf). AUC values were obtained using the linear trapezoidal rule and Thalf was estimated as the value 0.693 divided by the slope of the terminal phase of the Ln-transformed loperamide plasma concentration-time profile (slopes defined using no less than 3 consecutive points using a uniform weighting procedure. Several cases were encountered where Thalf could not be defined). These noncompartmental estimates were obtained using the Phoenix 64 WinNonlin 6.3 software program (Build 6.3.0.395). In addition to estimating the AUC from time zero to the last quantifiable concentration (AUC0-last), the area was also estimated from hrs 0-2 (AUC2) or hrs 0-5 (AUC5). Because our primary concern was extent of exposure, Cmax was indirectly evaluated by a comparison of the variability in the individual concentration vs time profiles.

Dose Proportionality:

The individual AUC values for all Collies at each loperamide dose were used to assess dose proportionality using the slope function in Excel. Statistical comparisons for dose proportionality were conducted three different ways, 1) using the original phenotype/genotype

groupings; 2) IVM sensitivity classification (sensitive = IVMS; non-sensitive = IVMNS), and 3), whether the Collies were classified as 3D or 4D dogs.

Statistical Analyses

Comparisons were generated using an analysis of variance (ANOVA) model (Proc GLM, SAS 9.3) by dose levels. The between animal classes comparisons focused either on genotype/phenotype, IVM sensitivity, or whether the Collie could tolerate only 3 or all 4 loperamide dose levels. Each ANOVA model included one of the three categorical covariates: genotype/phenotype, IVM sensitivity and 3D/4D. The Sidak multiplicative inequality was used to control maximum experiment-wise error rate under the set of null hypotheses related to the 4 categories of genotype/phenotype assuming positive dependence among the test statistics of interest. Statistical tests were two-sided with significance defined as $p < 0.05$.

Tabulated means and standard deviations provided in the tables reflect the simple arithmetic (untransformed) calculations.

An integral part of this study was the clinical signs observed in the Collies after each dose of loperamide. The PKPD comparative analyses were performed three different ways, 1) using the original phenotype/genotype groupings; 2) IVM sensitivity classification (sensitive = IVMS; non-sensitive = IVMNS), and 3), whether the Collies were classified as 3D or 4D dogs. As the multinomial clinical scores were ordinal in nature, they were analyzed using a cumulative logit model with exposure parameters (AUC₂, AUC₅ and AUC_{0-last}) as covariates and the group variables defined above as fixed effects. Note that we do not necessarily have to take the

ordering into account. However, ordinality in the response is vital information; ignoring it almost always will lead to sub-optimal models. Taking the natural ordering into account can lead a simpler, more parsimonious model and increase power to detect relationships with other variables. The cumulative logit model was implemented using the Proc Genmod (SAS 9.3) with generalized estimating equation (GEE) methods. Probability of the clinical score (y) less than or equal to category j ($j=0, 1, 2,$ and 3), $P(y \leq j)$, is of the interest rather than $P(y=j)$.

RESULTS

The variability in AUC_{0-last} values, expressed as the coefficient of variation (%CV), were nearly 2X greater in the Mut Collies compared to the WT dogs (Table 2, Figure 1). Variability of the heterozygotes (HNS and HS) tended to be greater than that seen with the WT but less than that associated with the Mut dogs. Similar differences in variability were seen in the IVMNS compared to the IVMS dogs (Table 2). No corresponding trends could be identified solely on the basis of dose. This genotypic/phenotypic difference in variability of AUC_{0-last} values was one of the most outstanding features of this dataset.

Because there was an observed relationship between the magnitude of drug exposure and the ability of the dogs to continue in the study through the final dose level, we further evaluated the exposure-response relationship by segregating the animals into 3D (Collies that could not be dosed > 0.1 mg/kg) and 4D groups (Collies that could tolerate all 4 doses). When divided in this manner, the 3Ds group consisted of Mut, HNS and HS dogs and the 4D group contained all four genotypes/phenotypes. When evaluating the time-concentration values for the 3D and 4D groups

over the first three doses of loperamide, the 4D Collies exhibited a lower intersubject variability (Figure 2). The 3D Collies exhibited a wider spread in their plasma loperamide concentrations at the 0.01, 0.05, and 0.10 mg/kg dose groups (Figs 2 & 3). Interestingly, while the IVMNS dogs tended to have lower loperamide exposure as compared to the IVMS dogs, the magnitude of this difference was substantially less than that seen between the 3D and 4D animals (Table 2 and Figure 4). Furthermore, a pairwise comparison of AUC₀-last values in HS vs HNS dogs revealed significant differences with respect to whether or not they displayed IVM sensitivity (Table 2).

In the absence of intravenous data, the impact of genotype and phenotype on loperamide pre and post absorptive processes cannot be differentiated. Accordingly, data were evaluated from the perspective of total PK drug characteristics using the AUC₀-last values (Tables 2). Statistically significant differences in WT vs Mut AUC₀-last values were achieved after the 0.1 mg/kg dose. However, when examining the mean concentration versus time profiles across all genotypes/phenotypes, a definite trend is seen with higher loperamide plasma concentrations being seen in the Mut versus the other classifications (Figure 5). The 3Ds had a statistically significantly higher total exposure as compared to that observed in the 4D dogs at all but the 0.05 mg/kg dose level (Table 2 and Figs 2 & 3), suggesting either a lower systemic clearance in the 3D group or a higher fraction of drug absorbed.

The dose proportionality of AUC₀-last value, expressed as the slope of the regression of AUC₀-last versus dose, was evaluated as a function of genotype/phenotype, IVM sensitivity and 3D/4D classification (Table 4). Statistically significant differences in slope were observed between the 3D and 4D dogs (4D Collies exhibited lower values). Differences in slope were also observed

between the WT and Mut dogs. No differences in dose proportionality were observed based on phenotype when comparing the IVMS vs IVMNS dogs (Table 4). The results of these evaluations are further illustrated by the graph of the dose versus loperamide exposure relationship as a function of genotype/phenotype (Figure 6) or 3D vs 4D dogs (Figure 7). In these graphs, the solid black line reflects the predicted AUC₀-last loperamide value based upon linear increases in exposure (estimated on the basis of the average concentration observed following a 0.01 mg/kg dose or, for the HNS dose group, the 0.01 mg/kg and 0.5 mg/kg dose group). The hatched lines represent the linear regression based upon the observed mean of the AUC₀-last values at each dose. Note that up through the 0.1 mg/kg dose, the concentrations of loperamide increased in a dose-proportional manner for all groupings. Nevertheless, the concentrations in the 3D dogs are substantially greater than those seen in the 4D animals.

The observed differences in loperamide concentrations could be attributable to either drug enterocyte permeability or to differences in presystemic drug loss. To further explore the potential contribution of drug metabolism, we considered the potential contribution of first pass drug loss. We measured the exposure (expressed as AUC₀-last values) for the primary metabolite, desmethyl loperamide (DML). If the reason for the higher loperamide exposure in the 3D vs 4D dogs was attributable to a reduction in its metabolic conversion, we would expect that the ratio of loperamide/DML AUC₀-last values would differ between these two groups. If such a difference is not observed, we would conclude that the higher levels of loperamide are associated with a higher permeability (greater intestinal absorption). As seen in Figure 8 (dose range 0.01 to 0.1 mg/kg), the vast majority of the 3D dogs were indistinguishable from their 4D counterparts. We did see two dogs did appear to have a higher loperamide/DML ratio.

Therefore, we further segregating the dogs into genotype/phenotype classification. Based upon this evaluation, there were no consistent differences in trends as a function of administered dose (Figure 9).

Of note is that when graphed as a function of genotype/phenotype, all groups exhibited a small negative trend in the loperamide/DML AUC₀-last ratios, indicating that as dose increased, the metabolite exposure tended to increase relative to that of the loperamide. A negative slope was observed in all but 5 dogs (3 Mut, 1 HNS, 1 HS; 4 = 4D; 1 = 3D). Since the loperamide and DML T_{max} values did not differ across doses (averaged within dose across all groups: loperamide T_{max} = 3.0, 2.6, 2.7, and 2.4; DML T_{max} values = 13.5, 14.6, 12.3 and 13 hrs after the 0.01, 0.05, 0.1 and 0.2 mg/kg doses, respectively), we cannot attribute this negative slope to a delay in the absorption as a function of dose. Moreover, since this trend was observed across all groups, we conclude that this observation was not associated with P-gp saturation. The lack of consistent differences in T_{half} values, regardless of grouping, along with the marked variability in these estimates (Table 3) further supports the contention that the differences in exposure are not attributable to mutation associated effects on drug clearance or volume of distribution. Thus, the higher plasma drug in 3D vs 4D dogs appears to be largely a function of the drug absorption process, with no apparent difference in the proportion of loperamide that undergoes first pass metabolism.

To ascertain whether there is a correlation between exposure and clinical response, AUC₀-last, AUC₂ and AUC₅ were evaluated. A lower correlation was observed between the clinical signs and AUC₀-last as compared to that associated with either AUC₅ or AUC₂ (data not shown).

Because most clinical signs were observed by hr 5 post-dose, all the analyses were performed using AUC5 as the exposure variable. Furthermore, upon examining all four clinical signs observed during this study (depression, ataxia, mydriasis and salivation), only ataxia and depression exhibited a phenotypic/genotypic distinction in exposure-response relationships. Therefore, subsequent assessments focused solely upon ataxia and depression versus AUC5.

The cumulative probability of less than or equal to category j ($j=0, 1, 2,$ and $3.$) of the clinical score for ataxia and depression were evaluated and plotted with regard to the various categorical variables (Figures 10-12). The Mut Collies had a much greater cumulative probability of exhibiting ataxia and depression not more than certain level of severity compared to the wild type, HNS or HS dogs. The exposures at which these responses occurred tended to be lower than that seen in any of the other groups, indicating a greater sensitivity to circulating loperamide. In addition, the Mut dogs had a greater propensity towards a higher severity of response (graded Level 0-3) in ataxia and depression when an adverse event was seen (Figs 10-11). Ataxia was associated with a greater risk at Level 2 or 3 scores as compared to that seen for depression.

HNS Collies had no incidents of depression, although several dogs exhibited Level 1 ataxia, with the probability of an event linked to drug exposure. Within the HS group, a similar AUC/response relationship for depression and ataxia was observed. Both of these signs occurred with a greater likelihood in HS compared to HNS dogs. In all cases, WT Collies had less than a 10% probability of exhibiting any sign of depression or ataxia but did exhibit mydriasis and salivation.

Depression and ataxia were observed in both 3D and 4D groups. However, the level of severity was less in the 4D Collies as compared to the 3D Collies. Furthermore, the 4D dogs were associated with a distinct right-shift of the exposure-response relationship both for ataxia and depression (Figure 12). Therefore, more drug exposures (lnAUC_{0_5}) were needed to achieve an adverse response in the 4D Collies compared to the 3D dogs. Accordingly, when considering effects from both the PK and the PK-PD relationship, the 3D dogs were associated with a greater overall risk of an adverse event being associated both with higher drug exposure and a greater likelihood of ataxia and depression at the lower drug exposures.

Five Mut Collies (2 in the 3D group, 3 in the 4D group) exhibited a score of 1 for only one of the clinical signs. In four of these five dogs, ataxia was observed. The fifth dog had a clinical sign of depression. This underscores the range of sensitivity to loperamide, even among the Mut genotype.

To further explore the sensitivity differences, we restricted our assessments to the Mut Collies in the 3D versus 4D groups. Within this group, for any AUC₅ value (i.e., across all dose levels), the likelihood of ataxia and depression was greater for Collies in the 3D group as compared to Collies in the 4D group. When these clinical signs were manifested, the 3D dogs trended towards a greater level of severity. Thus, irrespective of drug exposure, it would appear that the physiological effects of loperamide within the central nervous system (CNS) of the Mut 3D dogs differed from that of the Mut 4D dogs. Because all of these Collies had nonfunctional P-gp, it is highly likely that this difference simply reflected typical population variability in drug response.

Lastly, all IVMS Collies were compared to all IVMNS Collies to determine the prognostic implications of this phenotypic classification. The results demonstrated that if a Collie was IVMNS, there was a corresponding low probability that it would also exhibit ataxia (and nearly zero probability of depression) in response to loperamide (Table 5). However, the converse could not be assumed, and IVMS Collies (with the exception of the Mut dogs) could not be predicted to be sensitive to loperamide).

DISCUSSION

P-gp recognizes and effluxes a multitude of structurally and biochemically unrelated substrates (cyclic, linear, basic, uncharged, zwitterionic, negatively charged, hydrophobic, aromatic, nonaromatic, amphipathic) that range from a molecular weight of 250 to 4000 (Hodges et al., 2011). The impact of the P-gp mutation on human loperamide PK has been a subject of debate (Benet et al., 2004). The current investigation in dogs was unique in that it not only provided an opportunity to examine WT and Mut Collies, but it also evaluated heterozygous Collies, and distinguished this group of Collies on the basis of genotype and phenotype (response to IVM). This study also examined the influence of the ABCB1-1 Δ mutation on PK and PD as a function of dose, thereby elucidating potential factors that may be responsible for observed disparities in the literature.

Although the presence of a P-gp mutation affected the magnitude of loperamide exposure, the PKs remained dose-proportional, irrespective of whether this was evaluated on the basis of genotype/phenotype or 3D vs 4D. Therefore, the higher slope associated with the P-gp Mut dogs reflect the higher drug concentration at a given dose. To determine if differences in concentrations were a function of loperamide permeability or metabolism, we examined the ratio of loperamide/DML AUC_{0-last} values. To avoid bias attributable to differences in amount of loperamide absorbed, this issue was evaluated from the perspective of the ratio of loperamide/DML AU_{0-last} values. No differences in the loperamide/DML AUC_{0-last} ratios were seen as a function of genotype/phenotype or 3D vs 4D classification, indicating that the ABCB1- Δ 1 gene did not affect loperamide metabolism. Given the lack of differences in these ratios as a function of dog classification, it is concluded that it was differences in drug absorption

rather than clearance or first pass drug loss that was affected by the functionality of canine P-gp. This was further confirmed by the lack of difference in $T_{1/2}$ values,

It is important to note that loperamide conversion to DML (based upon human hepatic microsomes) has been associated not only with CYP3A4 but also with CYP2C8, CYP2B6 and CYP2D6 (Kalgutkar and Nguyen 2004; Kim et al., 2004). Accordingly, several metabolic pathways may be involved. Although loperamide undergoes first pass and hepatic metabolism (Baker, 2007), the substantial delay in DML T_{max} relative to that of loperamide is consistent with the majority of DML formation occurring after its first pass through the enterocyte or the liver. Accordingly, the observed differences between WT and Mut dogs are reflective of the influence of the P-gp mutation on drug absorption rather than on loperamide metabolism. In this regard, when and if functional P-gp enhances or reduces intestinal drug absorption remains a topic of debate (Benet et al., 2004, Tam et al., 2003; Dufek et al., 2013).

Another interesting observation was that for all but 5 dogs (all either homozygous or heterozygous for the ABCB1 Δ gene), the loperamide/DML AUC_{0-last} ratio tended to decrease as a function of dose. The similarity of loperamide and DML T_{max} values across all doses indicates that this change in ratio was unlikely a consequence of loperamide effects on gastrointestinal transit time. The late DML T_{max} observed in all dogs prohibited an evaluation of the elimination portion of the DML concentration-time profile. Nevertheless, considering the dose proportionality of loperamide systemic concentrations across all genotypes/phenotypes (and 3D vs 4D dogs), the dose-associated decrease in the AUC_{0-last} ratio is likely attributable to a reduction in DML elimination. Based upon data generated in human liver microsomes and rats,

loperamide is characterized by a complex set of metabolic pathways, with DML being its primary metabolite. (Kalgutkar and Nguyen, 2004). Saturation of any of these pathways could lead to increased DML concentrations. However, with respect to the relevance of this observation to the current investigation, we observed that this decrease in ratio was not influenced by the competency of dog P-gp.

Of particular note was not necessarily the impact of the mutation on mean systemic drug exposure but rather on the variability of drug exposure that occurred as a function of Collie classification. Loperamide exposure (expressed as AUC_{0-last} values) and the corresponding variability in that exposure in the Mut dog typically exceeded that of their WT counterparts. With the small number of subjects included in the investigation by Mealey et al., (2010), differences from that seen by Kitamura et al., (2008), the apparent interstudy disparity may simply be a function of “chance.” Moreover, we observed that those Collies trending towards a lower adverse response to loperamide (4D) also tended to exhibit lower plasma level variability as compared to that of the Mut or the 3D Collies. Reasons for this genotype/phenotype-associated higher variability (which we concluded is most likely attributable to the absorption phase of the PK profile) is a question worthy of further investigation.

With regard to the heterozygotes, the variability in AUC_{0-last} values ranged between that of the WT and Mut dogs. No significant differences could be detected between the AUC_{0-last} values of heterozygotes that were classified as HS vs HNS. With regard to their clinical response to loperamide, some of the dogs classified as IVMNS were found to be sensitive to loperamide-induced CNS toxicities, and some dogs classified as IVMS were non-sensitive to loperamide. As

with the Mut dogs, differences in response to loperamide compared to IVM is likely a function of the integrity of the blood brain barrier and also the inter-individual differences in drug response if the drug enters the brain. Thus, when comparing exposure-response toxicities across all four genotypes/phenotypes, it is important to consider: 1) the ability of loperamide to cross the blood brain barrier (i.e., if loperamide does not get across, it will not cause a toxic response); and 2) the pharmacology of loperamide within the CNS of the individual animal.

Considering the range of PK/PD characteristics seen in the heterozygotes, it would appear that the presence of the ABCB1-1 Δ mutation leads to a unique biology that needs to be explored further. From a therapeutic perspective, this variability can have tremendous implications with regard to the possible range of heterozygosity on disease expression (Bonneau et al., 2014). In other words, even if the trait is recessive, there may be other downstream consequences associated with being a carrier of just one recessive gene (e.g., Stribl et al., 2014). Potentially, this variability may also be associated with tissue-specific gene expression (Loeuillet et al., 2007, Zhang et al., 2009, Lo et al., 2003).

The complexity of the PK/PD responses to ABCB1-1 Δ mutation raises the question of the mechanism for the magnitude of variability within a given genotype. For example, does the ABCB1-1 Δ mutation influence the translation of genes other than those that encode P-gp? The rationale for this hypothesis relates to the intersubject variability observed in dogs expressing this mutation (e.g., Mut dogs). Could the remnant of this one gene influence the expression of other genes, or elicit some downstream epigenetic modifications that might normally occur if the gene did not possess a premature stop codon (e.g., Kim et al., 2014; Ingelman-Sundberg et al.,

2013)? If this is the case, then much of the work with knock-out mice (where the gene is totally removed) could lead to a biased study result whereby there is an inappropriate loss of ability to identify these “innocent victim” effects of the mutation.

For heterozygote dogs, it may also be worthwhile to consider whether there is differential tissue-specific expression of the functioning and/or non-functioning gene. Individuals heterozygous for the ABCB1 3435C>T polymorphism show differential expression of one or the other alleles at a single cell level (Loeuillet et al., 2007). Differences in allelic expression are also a common phenomenon elsewhere within the human genome (Lo et al., 2003). MRP2 knock-out mice exhibit an up-regulation in P-gp (Hoffman and Löscher, 2007). Thus, it is conceivable that Collies heterozygous for the ABCB1 Δ gene may have differential tissue expression of the mutant gene.

Further supporting this hypothesis are the multiple pathways controlling production and persistence of P-gp. P-glycoprotein gene expression and protein production are controlled at several discrete points in the regulatory pathways leading from DNA to expression of functional protein, with final protein levels a function of translational and post-translational/epigenetic control. P-gp expression is under the control of transcription factors (Kobori et al., 2014; Henrique et al., 2013; Chen & Sikic, 2012) which can be activated by a wide variety of agents (Cascorbi, 2011). Expression is also altered epigenetically through changes in DNA methylation or acetylation (Reed et al., 2010; Tomiyasu et al., 2014, Chen & Sikic, 2012) or changes in ubiquitination (Nawa et al., 2012; Zhang et al., 2004). Changes in P-gp expression can also be influenced by changes in the expression of other transporter genes and prior drug

exposure (Xia et al, 2009; Kobori et al., 2014). This epigenetic regulation may be of particular importance to dogs that are heterozygous for this trait.

When considering factors influencing gene expression, it is important to recognize the dynamic nature of cellular biology and the potential for alterations in transcription and translational processes over time. For example, with regard to P-gp, age-associated decreases in P-gp function appear to occur in elderly humans (Toornvliet et al., 2006). This decrease may not be uniformly expressed but rather localized in specific tissues. Using positron emission tomography, this decrease in P-gp activity observed in older human subjects appeared to preferentially influence the white matter of the brain (Bartels et al., 2010). Similarly, age-associated decreases in P-gp activity has been observed in the brains of rats (Silverberg et al., 2010), and dogs (Pekcec et al., 2011). In canine post-mortem tissue from 23 non-laboratory dogs, the dentate hilus and dentate gyrus indicated a 77 and 80% reduction respectively in dogs aged 37–99 months in comparison with younger individuals. In contrast, P-gp expression rates in the parahippocampal cortex increased with further aging in dogs with plaque formation (Pekcec et al., 2011). Given the results of our current investigation, it is interesting to consider whether decreased P-gp expression in the aged canine brain may be more apparent in heterozygous dogs as compared to their WT counterparts.

Ultimately, the results of this study underscore the complexity of effects that can be associated with genetic mutations and the need to consider this complexity when we try to extrapolate effects from one drug to another or from one animal model to the target species. As seen in this investigation, prior expression of sensitivity to P-gp substrates is suggestive, but not perfectly

predicted, of a higher risk of sensitivity to other P-gp substrates. While P-gp variants can be expected to potentially have a range of effects across therapeutic compounds, the ABCB1-1 Δ mutation is unique in that the resulting P-gp molecule is truncated and nonfunctional. Accordingly, one might suspect that all P-gp substrates would be comparably affected by this mutation. In other words, because the ABCB1-1 Δ mutation results in a truncated (nonfunctional) transporter, we anticipated that any drug whose entry into the CNS is restricted by P-gp would be similarly affected by the absence of this efflux transporter. However, given that sensitivity to IVM did not necessarily translate into a corresponding sensitivity to loperamide in Mut, HS and HNS dogs, it is likely that the therapeutic impact of this transporter defect will be drug specific, reflecting the inherent range of physiological effects that can occur within the CNS. Therefore, when P-gp substrates are administered to dogs that are potential carriers of this defect, animals should be carefully monitored for potential adverse effects, even if animals are heterozygous for the mutation. Importantly, we now recognize that there are genetic mutations other than the ABCB1-1 Δ mutation, that can lead to defective P-gp activity (Mizukami et al., 2013). It is incorrect to assume that the PK consequences of such defects will be uniform across all drugs.

Clearly, with respect to loperamide, with the exception of the WT dogs, sensitivity to IVM does not necessarily correctly identify those Collies that would express an adverse reaction to loperamide. In part, across genotype and phenotype, differences were observed both in terms of dose-exposure relationships and in terms of exposure-response relationships. With respect to dose-exposure relationships, the high variability observed in dogs carrying the genetic mutation suggest that there are other factors downstream from the P-gp itself that are influenced by the

presence of the ABCB1-1 Δ mutation. The mechanism for this additional effect cannot be discerned from this investigation but appears to influence loperamide presystemic PK characteristics.

In some ways, this research raised more questions than it answered. Thus, one of the important contributions of this work is the identification of questions that need to be considered whenever exploring potential implications of any mutation and genotype on the PK and exposure-response relationships.

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Wrote or contributed to the writing of the manuscript: Myers, Martinez, Li, Qiu, Troutman,
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Footnotes

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The views expressed in this article are those of the authors and do not necessarily reflect the official policy of the Department of Health and Human Services, the U.S. Food and Drug Administration, or the U.S. Government.

The experimental protocol was approved by the Animal Care and Use Committee at the Office of Research, Center for Veterinary Medicine, U.S. Food and Drug Administration, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (2011) and the Animal Welfare Act of 1966 (P.L. 89-544), as amended.

Figure Legends

Figure 1: Box plot comparisons of natural log-transformed AUC for Collies grouped by genotype & phenotype for all four doses used in this study. The four doses of loperamide used in this study were 0.01 mg/kg, 0.05 mg/kg, 0.10 mg/kg, and 0.20 mg/kg. See Table 1 for information on the sample size of each group.

Figure 2: Box plot comparisons of natural log-transformed AUC for Collies that received three (R) or Four (NR) doses of loperamide *in toto*. The box plots are the lnAUC values for the three lowest doses of loperamide used in this study; 0.01 mg/kg, 0.05 mg/kg, and 0.10 mg/kg. See Table 1 for information on the sample size of each group.

Figure 3: Time-concentration curves for the Collies when grouped by Collies that received the three lowest doses of loperamide (3D) versus Collies that received all 4 doses of loperamide. The results are grouped by treatment dose. The results shown are the mean values \pm SD.

Figure 4: Time-concentration curves for the Collies when grouped by sensitivity to exhibit ivermectin-induced CNS toxicities. The Collies are grouped into Ivermectin Sensitive Collies versus Ivermectin Non-Sensitive Collies, and further grouped by treatment dose. The results shown are the mean values \pm SD.

Figure 5: Time-concentration curves for the Collies when grouped by genotype & phenotype for the four doses of loperamide used in this study. The four doses of loperamide used in this study were 0.01 mg/kg, 0.05 mg/kg, 0.10 mg/kg, and 0.20 mg/kg. The results shown are the mean values \pm SD.

Figure 6: Loperamide AUC_{0-last} (expressed as ng*hr/mL) vs dose as a function of genotype/phenotype. The dashed line reflects the regression line derived on the basis of the observed values. The solid black line represents the expected relationship between dose and AUC_{0-last} in the presence of perfect dose proportionality. Note that in the HS dogs, the lighter dashed line reflects dose proportionality estimated on the basis of the 0.05 mg/kg rather than on

the 0.01 mg/kg dose. In all cases, the average exposure was proportional to dose, even though there were differences in the magnitude of the exposure across the various dog groups.

Figure 7: Loperamide AUC_{0-last} as a function of dose in 3D vs 4D dogs. The dashed line reflects the regression line derived on the basis of the observed values. The solid black line represents the expected relationship between dose and AUC_{0-last} in the presence of perfect dose proportionality.

Figure 8: Relative exposure, loperamide versus DML, expressed as the ratio of the respective AUC_{0-last} values estimated within each dog. Collies are classified on the basis of their adverse reaction to loperamide. Data are truncated to 0.1 mg/kg because this is the limit of drug administration in the 3D dosing group. Regression equation reflects trendline associated with the average loperamide concentration observed at each dose level (0.01 mg/kg to 0.1 mg/kg loperamide doses).

Figure 9: Relative exposure, loperamide versus DML, expressed as the ratio of the respective AUC_{0-last} values estimated within each dog: comparison of dogs across genotypes/phenotypes. Regression equation reflects trendline associated with the average loperamide concentration observed at each dose level

Figure 10: The probability that a given loperamide ln-AUC value will result in a CNS-induced ataxic score of 0, (black), 1 (blue), 2, (red), or 3 (green) is depicted. Clinical scores range from 0 (no response) to 3 (maximum response). Results are depicted by genotype.

HNS = Heterozygous, non ivermectin sensitive Collies (one normal ABCB1 gene and one ABCB1-1Δ gene).

HS = Heterozygous, ivermectin sensitive Collies (one normal ABCB1 gene and one ABCB1-1Δ gene).

Mut = Homozygous mutant Collies (two ABCB1-1Δ genes)

WT = Wild-Type Collies (two normal ABSVB1 genes)

Figure 11. The probability that a given loperamide ln-AUC value will result in a CNS-induced depression score of 0, (black), 1 (blue), 2, (red), or 3 (green) is depicted. Clinical scores range from 0 (no response) to 3 (maximum response). Results are depicted by genotype.

HNS = Heterozygous, non ivermectin sensitive Collies (one normal ABCB1 gene and one ABCB1-1 Δ gene).

HS = Heterozygous, ivermectin sensitive Collies (one normal ABCB1 gene and one ABCB1-1 Δ gene).

Mut = Homozygous mutant Collies (two ABCB1-1 Δ genes)

WT = Wild-Type Collies (two normal ABCB1 genes)

Figure 12: The probability that a given loperamide ln-AUC value will result in a CNS-induced ataxic score of 0, (black), 1 (blue), 2, (red), or 3 (green) is depicted. Clinical scores range from 0 (no response) to 3 (maximum response). Results are depicted by 3D Collies vs. 4D Collies.

HNS = Heterozygous, non ivermectin sensitive Collies (one normal ABCB1 gene and one ABCB1-1 Δ gene).

HS = Heterozygous, ivermectin sensitive Collies (one normal ABCB1 gene and one ABCB1-1 Δ gene).

Mut = Homozygous mutant Collies (two ABCB1-1 Δ genes)

WT = Wild-Type Collies (two normal ABCB1 genes)

Table 1: Collie Group Size, Ages, and Weights

Group	Group Size	Gender ratio (M/F)	Weight (kg)*	Age (years)*	IVM Sensitivity‡	3D [†] or 4D Collies per group
Wild-Type	7	4/3	26.4 ± 1.2	3.6 ± 0.7	0/7	0/7
Heterozygous Non-IVM Sensitive	9	2/7	28.4 ± 1.9	4.3 ± 0.9	0/9	1/8
Heterozygous IVM-Sensitive	7	3/4	26.1 ± 1.8	3.8 ± 0.7	7/7	3/4
Homozygous Mutant	10	5/5	27.1 ± 1.5	4.0 ± 0.4	10/10	6/4

The ABCB1 genotype and the IVM sensitivity (phenotype) of the Collies were known prior to enrollment in the study.

*Mean ± SEM

‡Number of Collies in group showing central nervous system toxicities following IVM administration (0.1 mg/kg p.o.) out of total number per group.

†Collies that received either 3 doses of loperamide (3D) or all 4 doses of loperamide (4D).

The 3D group received the 0.01 mg/kg, 0.05 mg/kg, and 0.10 mg/kg doses of loperamide. The 4D group received all 4 doses of loperamide; 0.01 mg/kg, 0.05 mg/kg, 0.10 mg/kg and 0.20 mg/kg.

Table 2: AUC_{0-last} Values for Each Loperamide Dose Analyzed by 3D vs. 4D, IVM Sensitivity/Nonsensitivity, or Genotype/Phenotype Grouping

Dose	Group	Mean	Stdev	n	%CV	Significant Diff*	p value
0.01	4Ds	2.61	0.98	22	37.55	3Ds v 4Ds	0.0066
	3Ds	4.13	1.76	10	42.62		
	WT	2.34	0.50	6	21.37		
	HNS	2.61	0.72	9	27.47		
	HS	3.49	1.44	7	41.26		
	Mut	3.67	2.01	10	54.77		
	IVMNS	2.59	0.64	15	24.71		
	IVMS	3.59	1.75	17	48.75		
0.05	4Ds	12.21	5.38	23	44.06		
	3Ds	17.30	9.80	10	56.65		
	WT	10.60	3.89	7	36.70		
	HNS	14.19	4.74	9	33.40		
	HS	10.95	5.11	7	46.67		
	Mut	17.52	10.37	10	59.19		
	IVMNS	12.62	4.63	16	36.69		
	IVMS	14.82	9.09	17	61.34		
0.1	4Ds	20.77	6.62	23	31.87	3Ds v 4Ds	0.0004
	3Ds	50.90	38.76	9	76.15		
	WT	18.98	6.70	7	35.30	mut v wt	0.0309
	HNS	29.03	19.64	9	67.65		
	HS	20.93	6.58	6	31.44		
	Mut	41.60	37.43	10	89.98		
	IVMNS	24.63	15.82	17	64.23		
	IVMS	33.85	31.01	15	91.61		
0.2	4Ds	50.50	34.03	23	67.39	NA	
	3Ds			0			
	WT	40.55	13.68	7	33.74	HNS v mut	0.0469
	HNS	46.49	13.52	9	29.08		
	HS	35.15	9.20	3	26.17	HS v mut	0.0186
	Mut	88.45	71.70	4	81.06		
	IVMNS	43.89	13.46	16	30.67		
	IVMS	65.61	58.40	7	89.01		

* P-values are shown only for group comparisons with statistically significant differences. All group comparisons were calculated.

Table 3: Mean Thalf Estimates for Each Loperamide Dose Analyzed by 3D vs. 4D, IVM Sensitivity/Nonsensitivity, or Genotype/Phenotype Grouping

Dose	Group	Mean	n	%CV
0.01	4Ds	10.6	22	48.3
	3Ds	11.4	10	45.9
	WT	10	6	42.4
	HNS	11.3	9	57.3
	HS	7.2	7	28
	Mut	13.5	10	35.5
	IVMNS	11.2	15	48.7
	IVMS	10.7	17	46.3
0.05	4Ds	12.3	23	30
	3Ds	21.3	10	92.5
	WT	10.6	7	33.9
	HNS	12.9	9	46.8
	HS	11.8	7	17.5
	Mut	24.7	10	81.1
	IVMNS	11.8	16	40.8
	IVMS	18.9	17	88
0.1	4Ds	12.7	23	
	3Ds	14.6	9	54.3
	WT	10.2	7	38.4
	HNS	14.3	9	40.5
	HS	16	6	80.3
	Mut	15.6	10	46.9
	IVMNS	15.1	17	36.7
	IVMS	15.2	15	100
0.2	4Ds	12.5	23	42.7
	3Ds		0	
	WT	10.4	7	26
	HNS	12.3	9	37.7
	HS	16.3	3	69.1
	Mut	13.9	4	24.7
	IVMNS	11.7	16	32.8
	IVMS	13.9	7	54.3

P-values are shown only for group comparisons with statistically significant differences. All group comparisons were calculated. No significant differences exist between any of the groups in this table

Table 4: Loperamide Dose Proportionality Values (expressed as slope of the regression of AUC_{0-last} vs dose) for Each Loperamide Dose Analyzed by 3D vs. 4D, IVM Sensitivity/Nonsensitivity, or Genotype/Phenotype Grouping

Group	Mean	Stdev	n	Significant Diff*	p value
4Ds	250.20	177.16	23	3Ds v 4Ds	0.012
3Ds	515.90	398.14	10		
WT	198.01	71.73	7	wt v mut	0.013
HNS	284.32	214.10	9		
HS	239.48	106.47	7		
Mut	529.24	414.81	10		
IVMNS	246.56	168.70	16		
IVMS	409.93	350.21	17		

*P-values are shown only for group comparisons with statistically significant differences. All group comparisons were calculated.

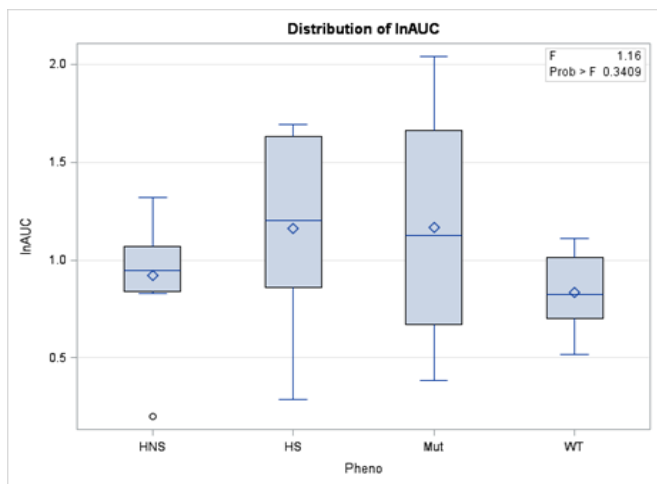
Table 5 Clinical Signs of Loperamide CNS Toxicity Observed in IVM Nonsensitive and IVM Sensitive Collies

Group	Clinical Sign (# of Collies)	
IVM Non Sensitive Collies*	Ataxia	(1)
	Salivation	(8)
	Depression	(0)
	Mydriasis	(4)
IVM Sensitive Collies*	Ataxia	(10)
	Salivation	(13)
	Depression	(6)
	Mydriasis	(11)

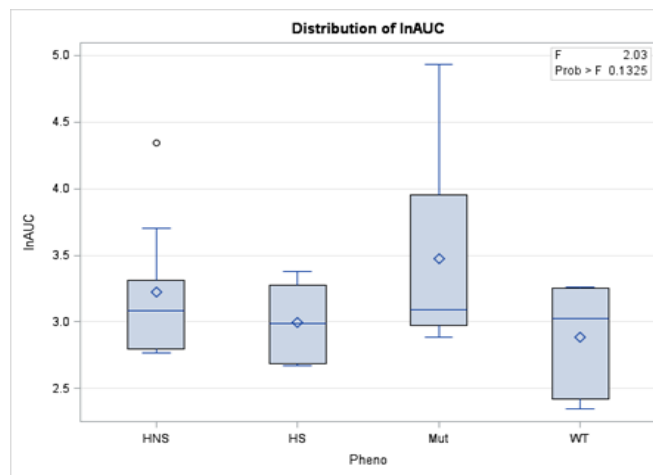
*Compilation of the clinical signs in all Collies across all dose groups.

Figure 1.

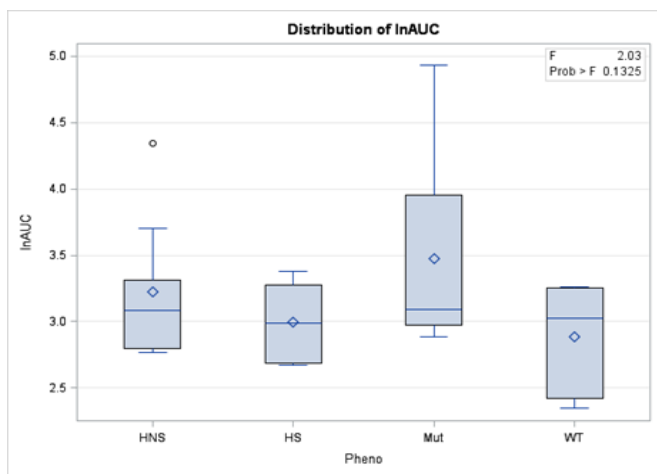
Dose = 0.01 mg/kg



Dose = 0.1 mg/kg



Dose = 0.05 mg/kg



Dose = 0.2 mg/kg

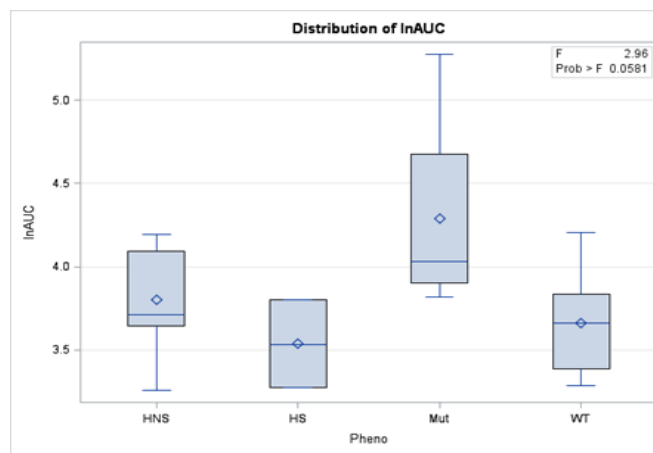
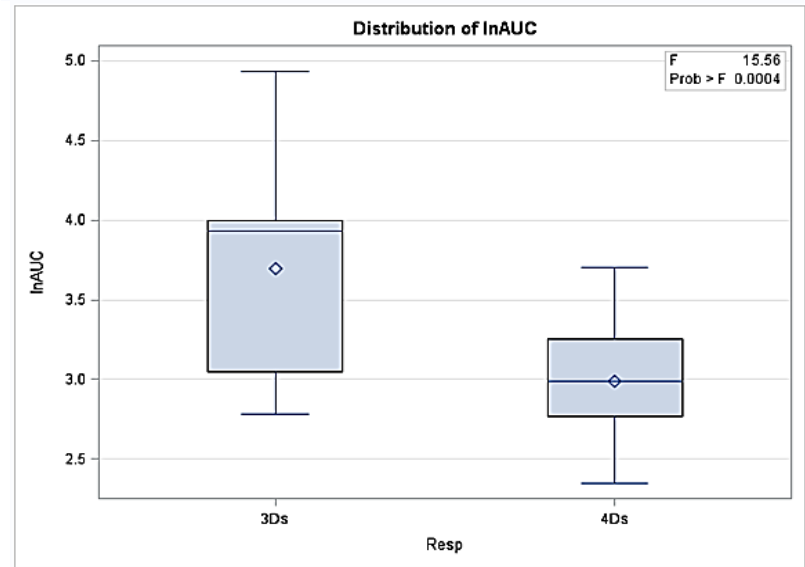
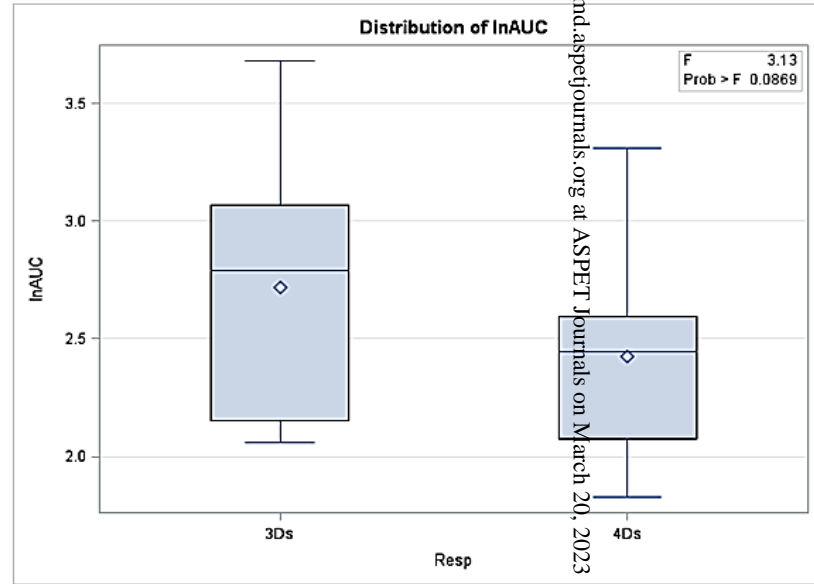
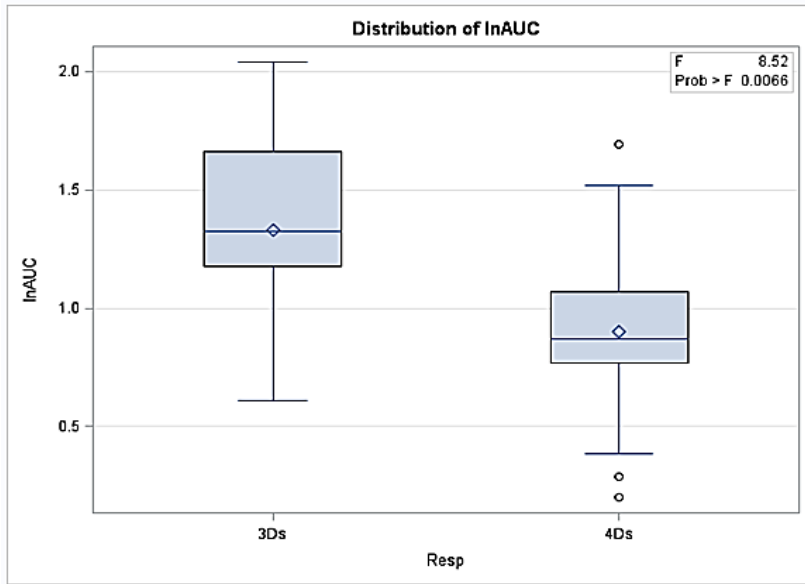


Figure 2

Dose = 0.01 mg/kg



Dose = 0.1 mg/kg

Figure 3

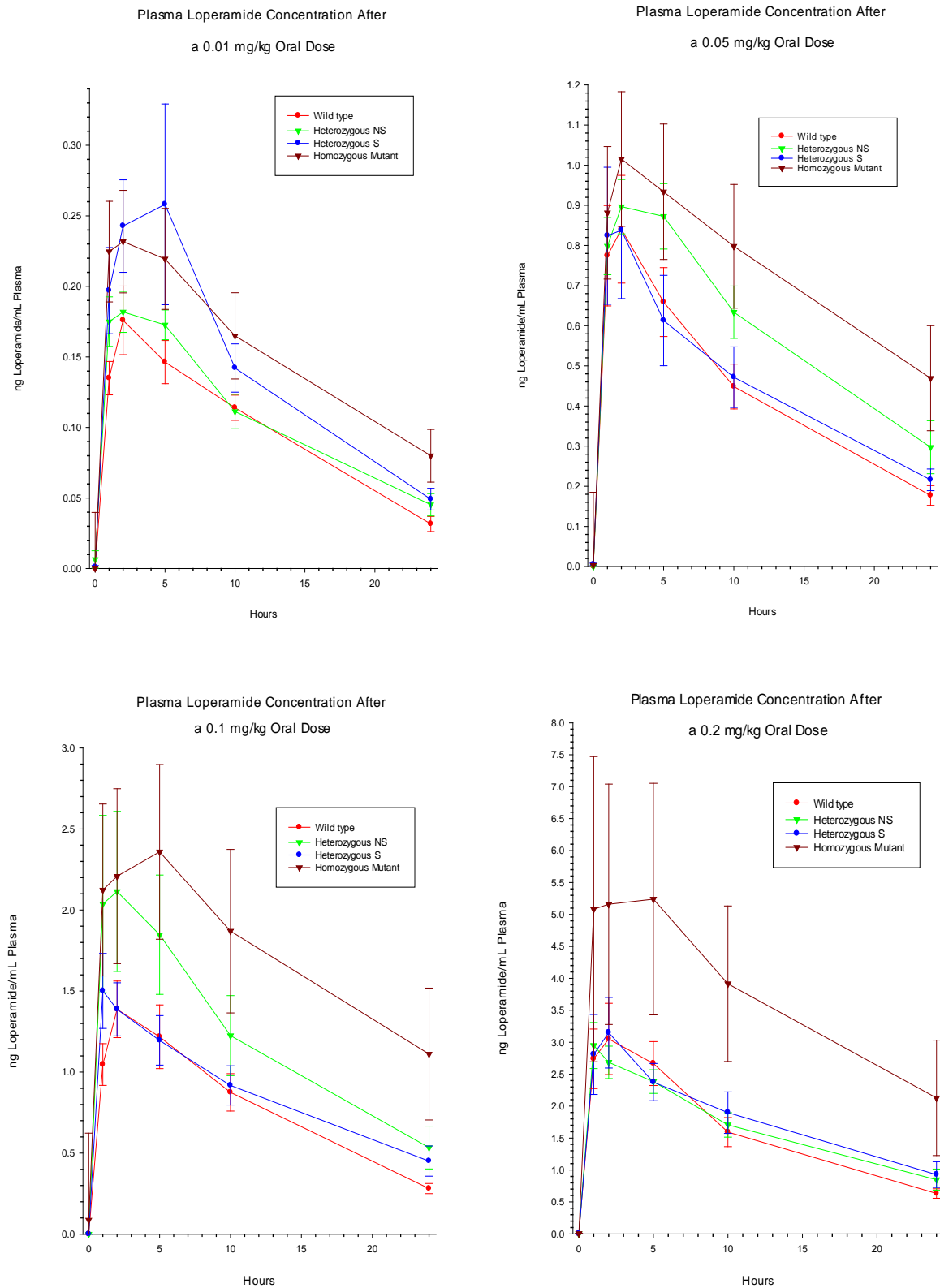


Figure 4

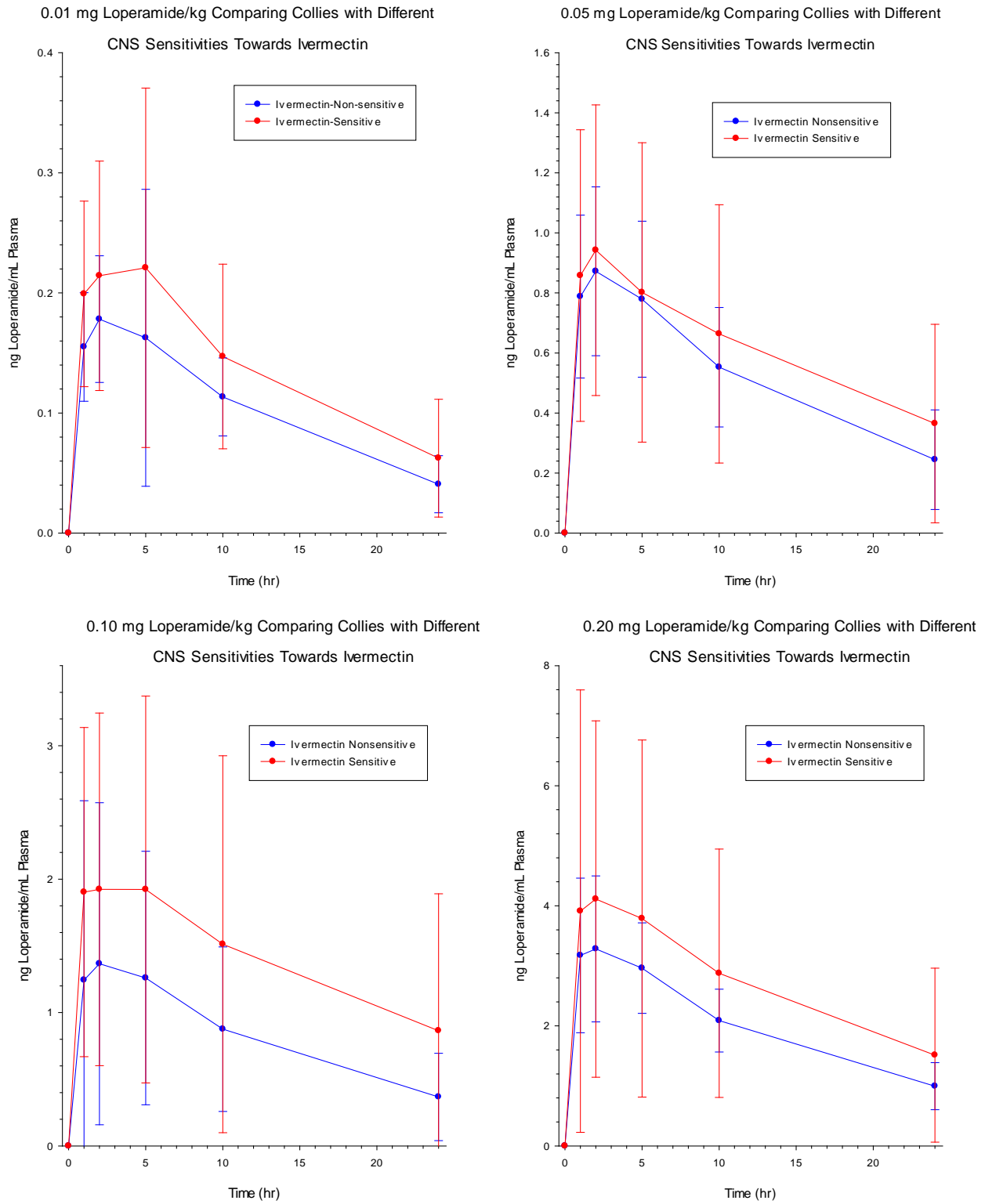


Figure 5

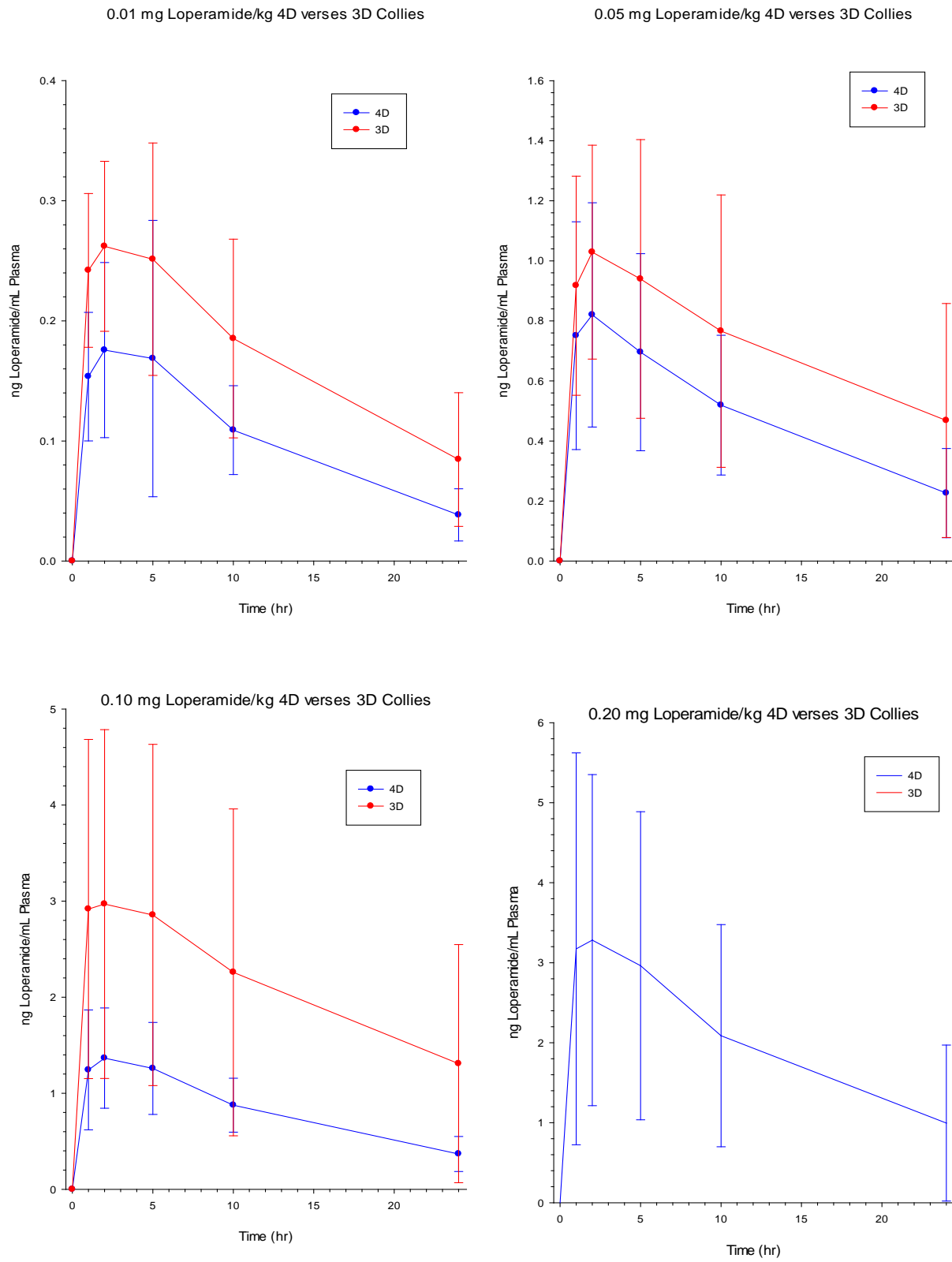


Figure 6.

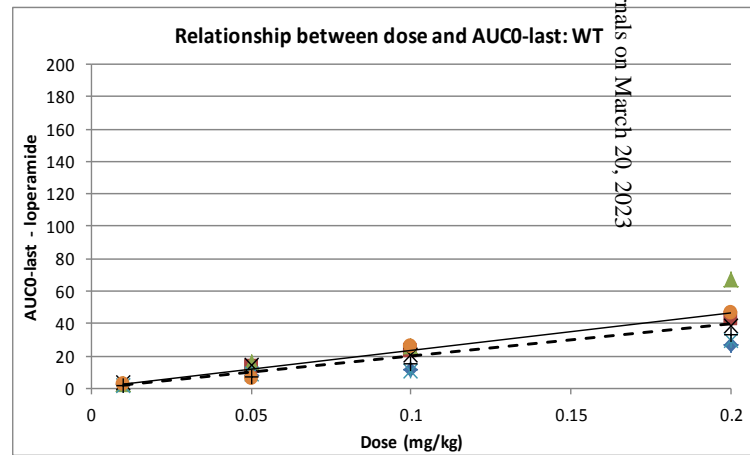
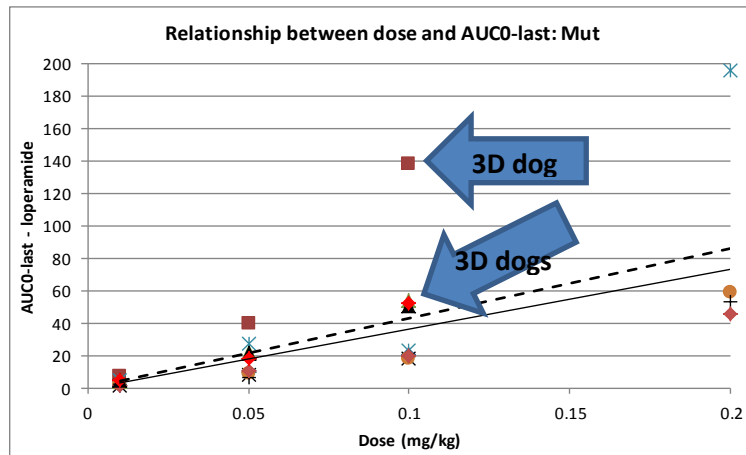
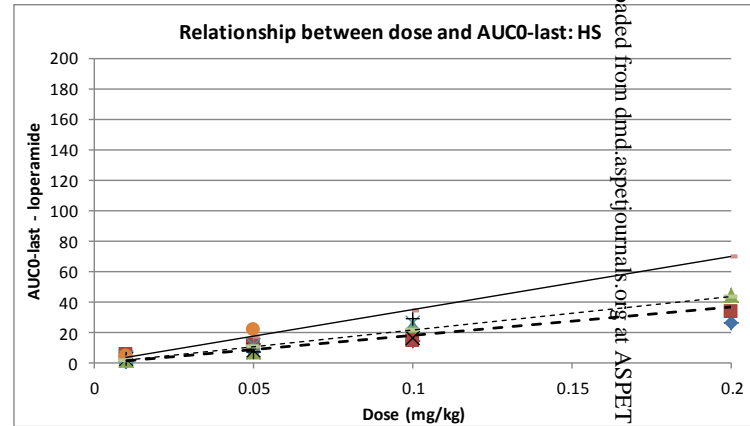
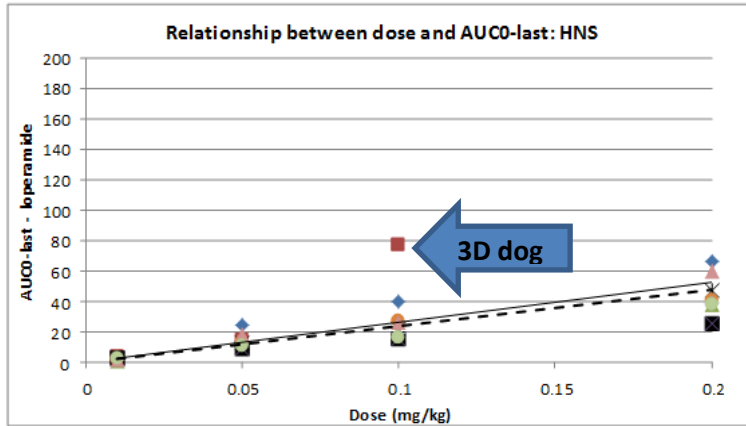


Figure 7

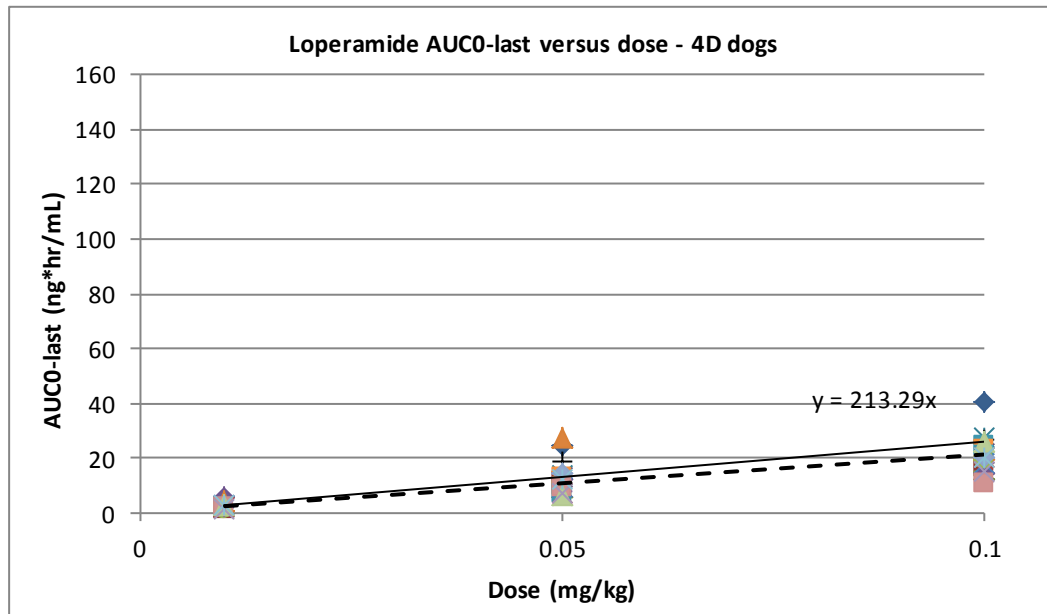
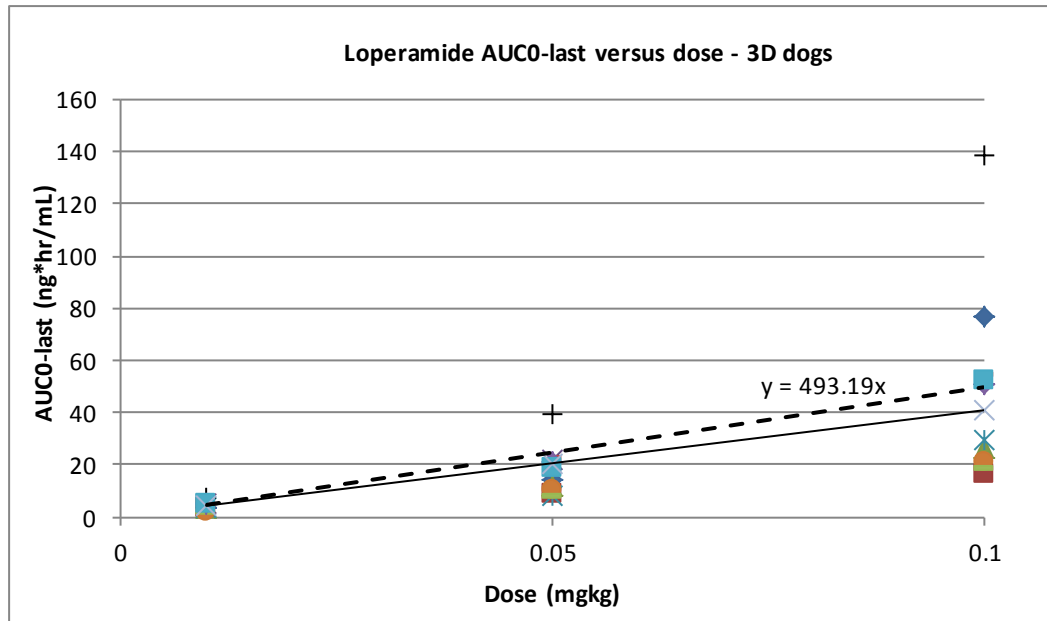


Figure 8

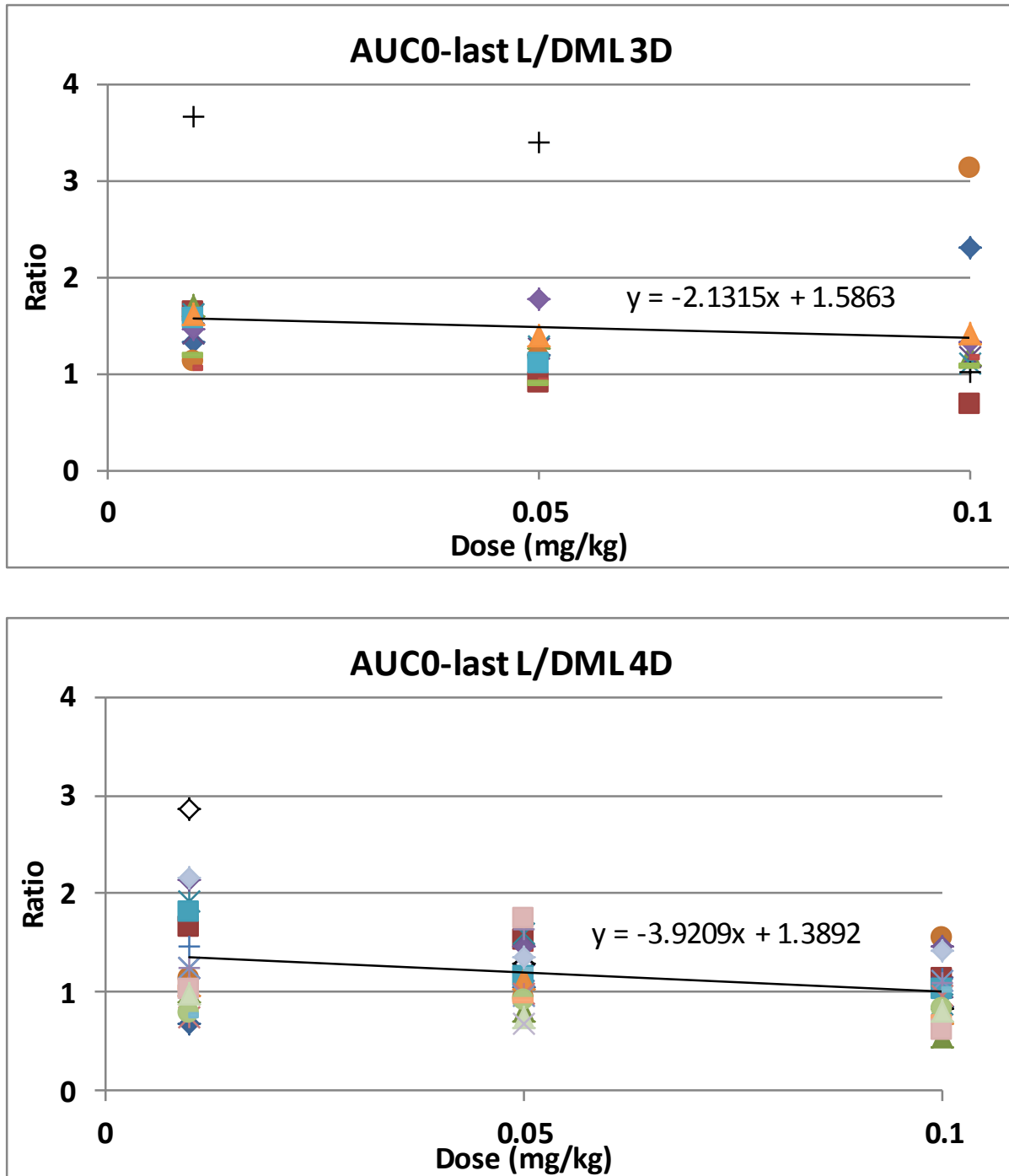
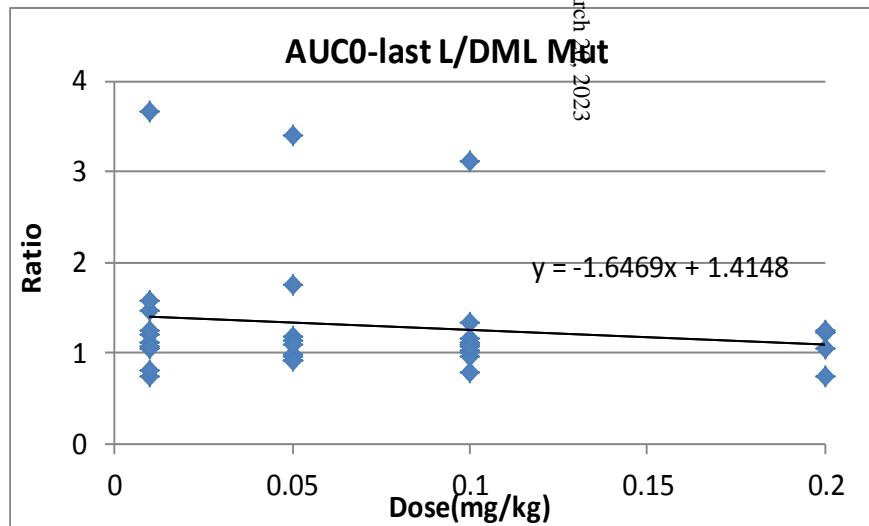
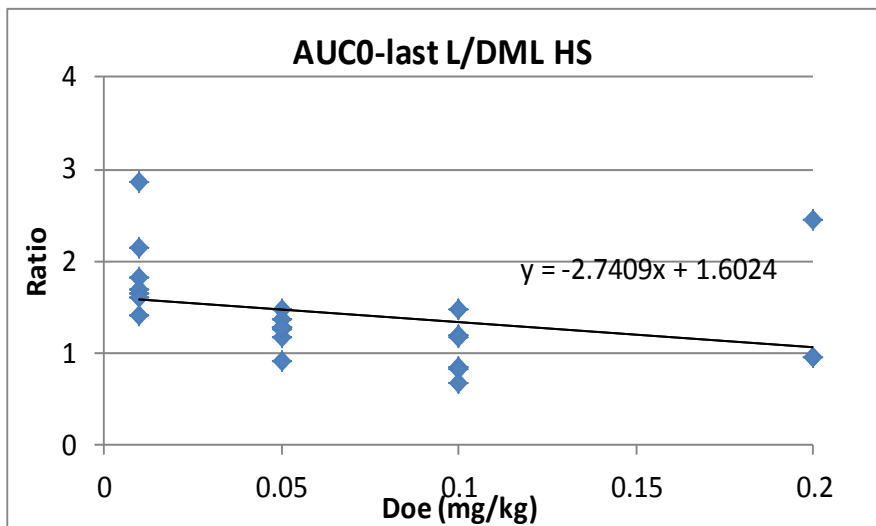
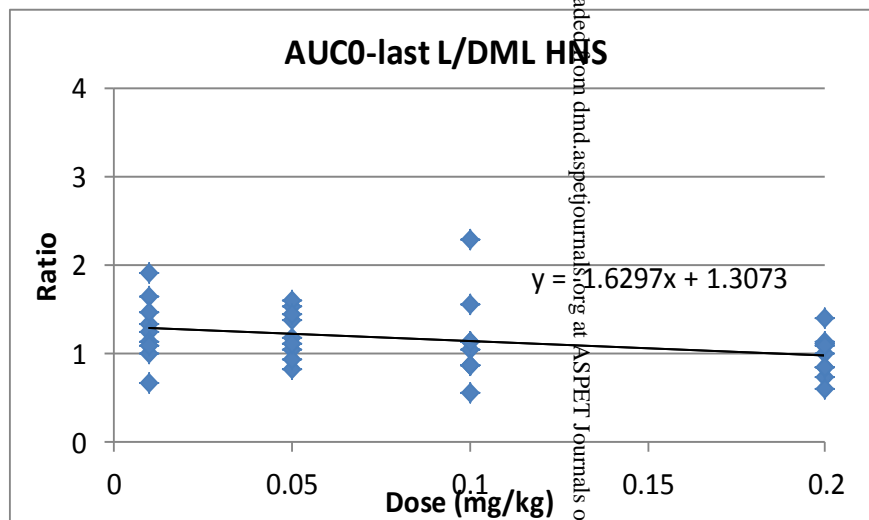
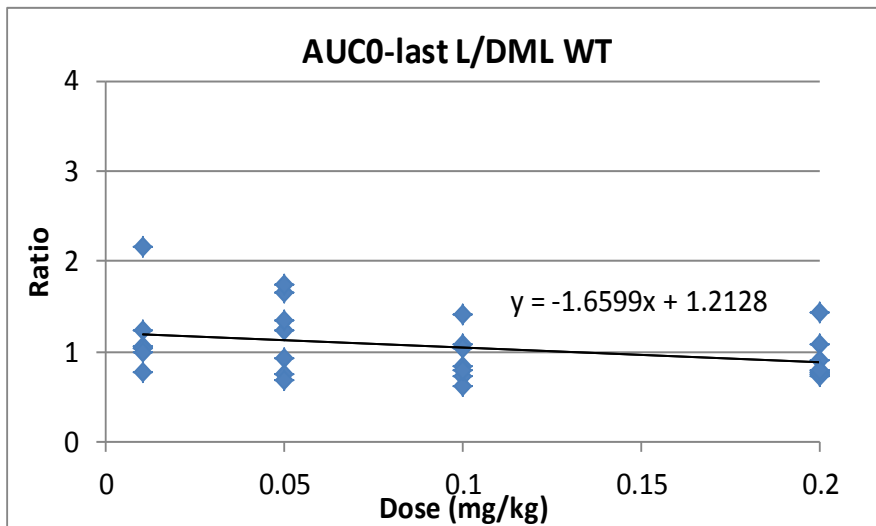


Figure 9



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Figure 10

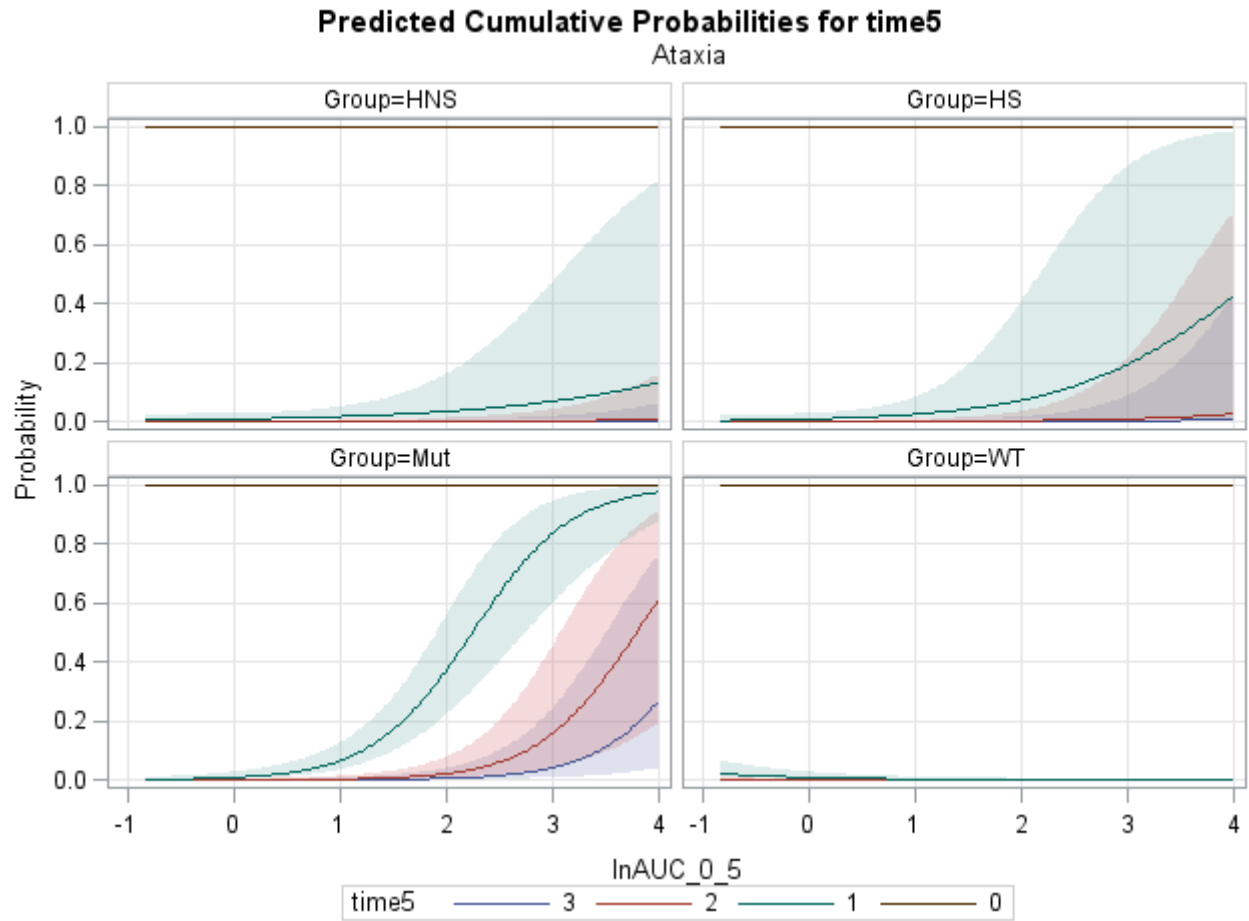


Figure 11

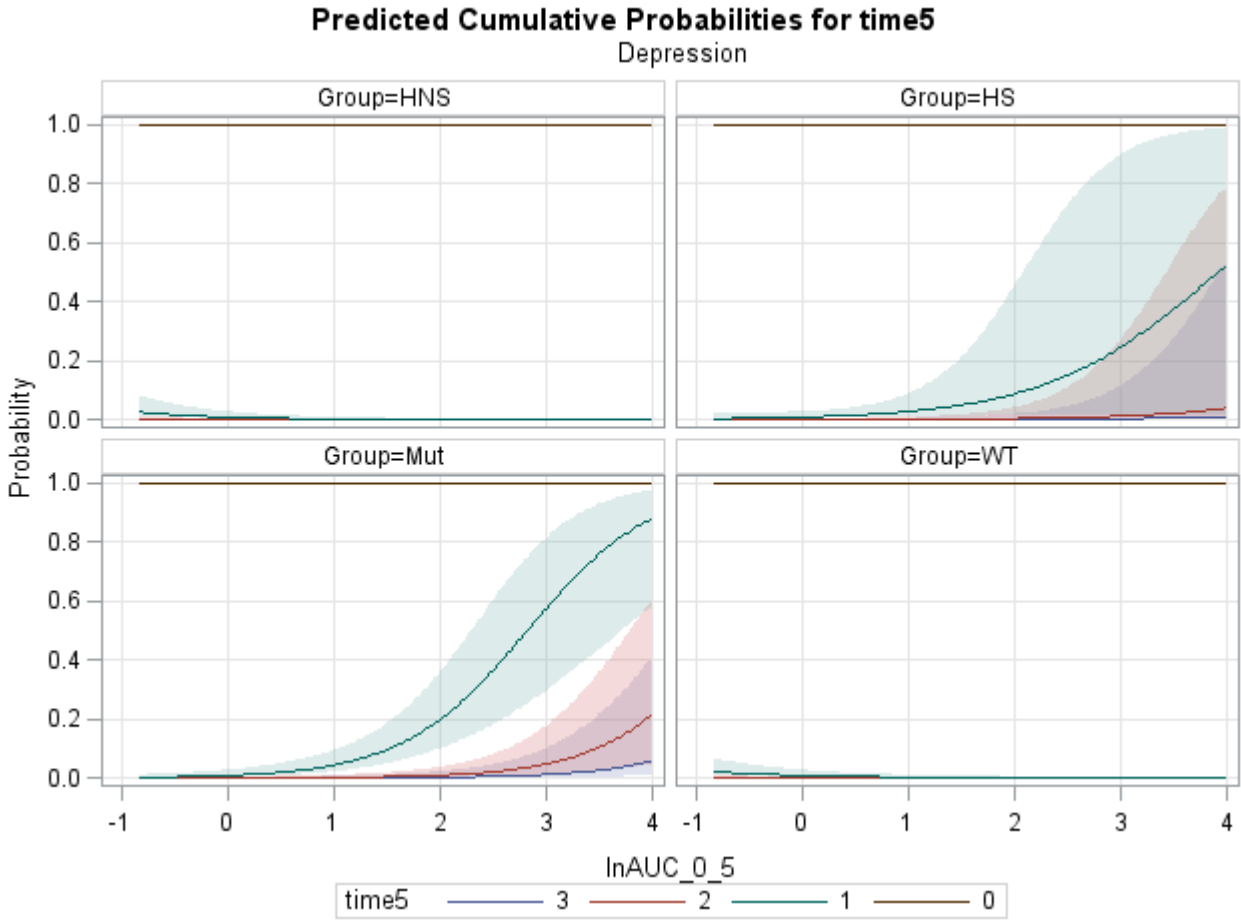


Figure 12

