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Canine albumin polymorphisms and their impact on drug plasma protein binding^a

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Counts:

Text pages: 26

Tables: 4

Figures: 4

References: 16

Abstract words: 250

Introduction words: 296

Discussion words: 1562

Nonstandard abbreviations: ACN – acetonitrile; cDNA – complementary deoxyribonucleic acid; C_{max} – maximum serum concentration; CV – coefficient of variation; DNA – deoxyribonucleic acid; HPLC – high performance liquid chromatography; MeOH – methanol; PCR – polymerase chain reaction; QC – quality controls; SD – standard deviation; SNPs – single-nucleotide polymorphisms; LOD – limit of detection; LLOQ – lower limit of quantitation; MS – mass spectrometry.

ABSTRACT

Drug binding to plasma proteins is routinely determined during drug development. Albumin polymorphisms c.1075G>T (p.Ala359Ser) and c.1422A>T (p.Glu474Asp) were previously shown to alter plasma protein binding of a drug candidate (4-[1-[3-chloro-4-[n'-(2-methylphenyl)ureido]phenylacetyl]-(45)-fluoro-(2S)-pyrrolidine-2-yl]methoxybenzoic acid, D01-4582) in a colony of Beagles. Our study investigated the hypothesis that drug-protein binding in plasma from dogs with albumin H1 (reference) allele would be greater than in plasma from albumin H2 allele dogs (c.1075G>T and c.1422A>T) (n = 6 per group). Plasma protein binding extent of four drugs (D01-4582, celecoxib, mycophenolic acid and meloxicam) was evaluated using ultracentrifugation or equilibrium dialysis. Free and total drug concentrations were analyzed by liquid chromatography–mass spectrometry. The albumin gene coding region was sequenced in 100 dogs to detect novel gene variants, and H1/H2 allele frequency determined in a large and varied population (n = 1446 from 61 breeds and mixed-breed dogs). For meloxicam, H1 allele plasma had statistically significant higher free drug fractions (P = 0.041) than H2 allele plasma. No significant difference was identified for plasma protein binding of D01-4582, celecoxib or mycophenolic acid. c.1075G>T and c.1422A>T were the most common SNPs in canine albumin, present concurrently in most study dogs and occasionally identified independently. Our findings suggest a potential influence of c.1075G>T and c.1422A>T on plasma protein binding. This influence should be confirmed *in vivo* and for additional drugs. Based on our results, albumin genotyping should be considered for canine research subjects to improve interpretation of pharmacokinetic data generated during the drug development process for humans and dogs.

INTRODUCTION

For some drugs, the risk of adverse effects (toxicity or lack of efficacy) depends on the concentration of free drug achieved in plasma. In some cases, free drug concentrations and the extent of drug binding to plasma proteins can be affected by differences in the albumin genotype. Canine albumin polymorphisms c.1075G>T (p.Ala359Ser; rs852211303) and c.1422A>T (p.Glu474Asp; rs851238996)^b have been identified as a cause of pharmacokinetic variability of a drug candidate (4-[1-[3-chloro-4-[n'-(2-methylphenyl)ureido]phenylacetyl]-(45)-fluoro-(2S)-pyrrolidine-2-yl]methoxybenzoic acid, D01-4582) in Beagle dogs (Ito et al., 2009). Specifically, dogs homozygous for both single nucleotide polymorphisms (SNPs) had up to 6-fold greater unbound drug fraction when compared to wild-type dogs, resulting in significantly greater drug clearance. While this report (Ito et al., 2009) identified an association between canine albumin genotypes and high inter-individual variability in drug disposition, the study was limited to only one experimental drug in a colony of research Beagles. The impact of canine albumin polymorphisms on plasma protein binding of other drugs and in a more heterogeneous dog population (outside of research colonies) remains to be investigated.

The primary objective of our study was to investigate the hypothesis that plasma samples collected from dogs with the albumin H1 allele (reference) have a higher extent of drug-protein binding than that of plasma collected from dogs with the albumin H2 allele (c.1075G>T and c.1422A>T). To test our hypothesis, we compared the extent of plasma protein binding of four highly albumin-bound drugs (D01-4582, meloxicam, celecoxib and mycophenolic acid) in plasma obtained from dogs with H1 and H2 alleles. Secondary

objectives of our study were to screen for other nonsynonymous variants in the coding region of the canine albumin gene that could influence protein tertiary structure, and to describe c.1075G>T and c.1422A>T genotype frequencies among a large number of different dog breeds.

MATERIALS AND METHODS

Reagents and chemicals

Mycophenolic acid, meloxicam and celecoxib, as well as their deuterated forms (mycophenolic acid-d₃, meloxicam-d₃ and celecoxib-d₄) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). D01-4582 was synthesized and generously provided by the Medicinal Chemistry Research Laboratory, Daiichi Sankyo (Tokyo, Japan). Chemical structures of each analyte and deuterated form are included in Figure 1. High-performance liquid chromatography-grade methanol (MeOH) and acetonitrile (ACN) were from Fisher Scientific (Pittsburgh, PA, USA). Milli-Q water of at least 18 MΩ resistance was used to prepare the mobile phase. Potassium phosphate monobasic (99%) was from Fisher Chemical (Fair Lawn, NJ, USA). Sodium chloride (99%) and potassium chloride (99%) were from Fisher Bioreagents® (Fair Lawn, NJ, USA). Sodium phosphate dibasic and formic acid (88%) from J.T. Baker (Phillipsburg, PA, USA).

Canine plasma samples

Blood samples were collected using procedures approved by the Washington State University (WSU) Institutional Animal Care and Use Committee (ASAF # 04662-004 and 04797-001). Owners provided written consent for their dogs' participation in the study. Pooled canine plasma collected into sodium citrate tubes from clinically healthy dogs (n = 6) was used for initial analytical assay validation (methods described below).

Sample size requirement to evaluate the effect of albumin genotype (H1/H1 versus H2/H2) on drug binding in plasma was determined based on mean and standard deviation results from a previous report (Ito *et al.*, 2009). This analysis indicated that a minimum of 5 dogs per group was needed to achieve an alpha value of 0.05 with a power value of 0.80 for a 6-fold difference in mean free drug fraction. Therefore, we proposed to study 6 dogs per genotype group.

Privately-owned dogs were recruited from the WSU student, faculty and employee population, and genotyped for the c.1075G>T and c.1422A>T variants (method described below). After initial screening, 6 dogs with the H1/H1 genotype (c.1075G and c.1422A) and 6 dogs with the H2/H2 genotype (c.1075T and c.1422T) were selected for our study. Breeds included in the H1/H1 genotype group: Labrador Retriever (n = 3), Afghan Hound (n = 1), English Pointer (n = 1) and Mixed breed (n = 1). Breeds included in the H2/H2 genotype group: Golden Retriever (n = 2), Borzoi (n = 1), Pitbull (n = 1), German Shorthair Pointer (n = 1), and Mixed breed (n = 1). All study dogs (5 castrated males and 7 spayed females) were judged to be clinically healthy based on history and physical examination, with no medications received for at least 14 days prior to blood collection. Owners were asked to fast the dogs on the morning of blood sample collection. Blood samples were collected by venipuncture into tubes with sodium citrate as an anticoagulant. Plasma was obtained by centrifugation (1800 x g for 8 min) of blood samples, transferred to cryotubes and stored at -80°C until analysis.

Determination of the extent of plasma protein binding of D01-4582, celecoxib, mycophenolic acid and meloxicam

D01-4582 was included in our study because of its reported binding differences associated with canine albumin genotype. The remaining compounds were selected based on their reported high plasma protein binding (> 95%), site-specific binding to human albumin (Er et al., 2013), and previous clinical use in dogs (Table 1). The method (ultracentrifugation or equilibrium dialysis) used for determining extent of each drug plasma binding was primarily selected based on methods previously reported for that drug (Ito et al., 2009; Morassi et al., 2018; Lockwood et al., 1983; Jolliet et al., 1997; Lapique et al., 2000; Cooper et al., 2014).

To control the potential effect of pH differences on drug protein binding, individual plasma samples were adjusted with 1N HCl to achieve a pH of 7.34 ± 0.04 (mean \pm SD) (Mettler Toledo, Columbus, OH, USA). The volume of acid used in these adjustments was less than 0.5 % of the total mixture. Plasma albumin concentration for each genotyped plasma sample was determined using a standard bromocresol green method, monitoring the absorbance at 630 nm.^c

Ultracentrifugation (for D01-4582)

Initially, 10 μ L of D01-4582 standard solution (2500 μ g/mL) was added to 490 μ L of plasma for a final concentration of 50 ppm (92.6 μ M). Samples were then incubated for 30 min at 37°C and 210 μ L of each plasma sample was centrifuged in a 0.5 mL thick-wall polycarbonate tube on a fixed-angle rotor Thermo Sorvall MX-150 Micro-Ultracentrifuge (Sorvall Instruments, Newton CT, USA) at 200000 x g for 16 h at 4°C. The ultracentrifugation resulted in visually distinct layers: superficial (lipid), middle (clear) and deep (protein pellet). To minimize disruption of the lipid superficial layer, the supernatant sample was collected from the middle layer immediately after centrifugation, as

recommended (personal communication with Dr. Takashi Ito). In addition, the rotor was kept in ice during collection, to maintain sample temperature. A 50 μ L aliquot of supernatant was collected into a 0.6 mL Eppendorf tube and prepared for HPLC-MS/MS analysis, as described below. Unbound fraction of D01-4582 was estimated by dividing the unbound concentration by the nominal incubation concentration. Plasma from a non-genotyped dog was used to confirm acceptable inter- and intra-run precision (CV<15%). The experiments for each dog were conducted in duplicate and repeated on 3 separate days.

High-throughput equilibrium dialysis (for celecoxib, mycophenolic acid and meloxicam)

A high throughput equilibrium dialysis device (96-well, 75- μ L half-cell capacity) and cellulose membranes (12–14 kDa molecular weight cutoff) (HT-Dialysis, Gales Ferry, Connecticut) were used to determine the extent of protein binding for celecoxib, mycophenolic acid and meloxicam. Dialysis membranes and device were conditioned and assembled as recommended by the manufacturer. Plasma samples were spiked with celecoxib (4 μ M), mycophenolic acid (12.5 μ M) and meloxicam (1.6 μ M).

Compound spiked plasma samples were transferred to the plasma (donor) chambers of the dialysis device (75 μ L per half-well), and an equal volume of phosphate buffer (15 mM, pH 7.3 \pm 0.1) was placed in the buffer (receiver) chambers. The dialysis device was sealed using the kit adhesive (HT-Dialysis), and dialysis was conducted in an orbital shaker (100 rpm) maintained at 37°C. Complete equilibrium was achieved in the HT-Dialysis apparatus within 3.5 hours (data not shown) and samples collected at the end of 4 hours. Samples were then processed for quantification by HPLC-MS/MS analysis as described below.

The extent of plasma protein binding of each analyte in all the plasma samples was estimated using the following mathematical expressions:

$$\% \text{ Bound} = (\text{Cb}/\text{Cp}) \times 100\%$$

$$\% \text{ Unbound} = 100 - \% \text{ Bound}$$

where Cb and Cp represent the measured concentration of the compound from the buffer and plasma chambers of the dialysis device, respectively.

Quality controls using mycophenolic acid (12.5 μM) (previously validated binding evaluation by equilibrium dialysis in canine plasma) were included in each row of the dialysis apparatus to monitor dialysis membrane integrity and assay performance. Experiments for each dog and compound were conducted in triplicate and repeated on 3 separate days.

Determination of drug concentrations

Chromatographic conditions

The concentration of each analyte was determined by liquid chromatography with mass spectrometry detection using a HPLC apparatus (Agilent 1100, Agilent Technologies, Santa Clara, CA) connected to a triple quadrupole mass spectrometry detector (AB-Sciex API4000, Applied Biosystems Life Technologies, Framingham, MA) operated in positive ion mode. Mobile phase consisted of 95% v/v water (containing 0.1% v/v formic acid) and 5% v/v acetonitrile at 1 mL per minute through a 2.1 mm x 50 mm 5 μm C18 column (Zorbax Eclipse XDB-C18, Phenomenex, Torrance, CA). Mass transitions monitored included m/z 540.3 \rightarrow 402.2 (D01-4582), m/z 382.2 \rightarrow 362 (celecoxib), m/z 386.2 \rightarrow 366 (celecoxib-d4), m/z 321.2 \rightarrow 207.1 (mycophenolic acid), m/z 324.2 \rightarrow 210.1 (mycophenolic acid-d3), m/z 352.1 \rightarrow 115.1 (meloxicam) and m/z

355.1→115.1 (meloxicam-d3). Retention times for D01-4582, celecoxib, mycophenolic acid and meloxicam were 2.20, 2.41, 2.11 and 2.14 minutes, respectively.

Calibration standards

Calibration standards and quality control (QC) samples (n = 3 for each concentration) were prepared in canine plasma prior to protein precipitation (for celecoxib, mycophenolic acid and meloxicam) or supernatant (D01-4582). The concentration ranges used for calibration curves and QC for each analyte are listed in Table 2.

Sample preparation for HPLC-MS/MS analysis

For D01-4582 samples, methanol (50 μ L) containing the internal standard (meloxicam-d3) was added to the supernatant sample (50 μ L). The mixture was vortexed and centrifuged at 10000 \times g for 15 min at 4°C, with the supernatant (20 μ L) introduced into the HPLC-MS/MS.

For celecoxib, mycophenolic acid and meloxicam samples, each 50 μ L aliquot of plasma (calibration standard or QC samples) was transferred into a 2 mL Eppendorf tube with 50 μ L of phosphate buffer, 1000 μ L of precipitating solution (ACN for celecoxib and meloxicam; MeOH:ACN 1:1 v/v for mycophenolic acid), and the respective internal standard (4 μ L for celecoxib-d4, 3 μ L for mycophenolic acid-d3 and 5 μ L for meloxicam-d3). For dialysis samples, the matrix was equilibrated prior to protein precipitation, by adding 50 μ L of plasma or phosphate buffer to the corresponding sample (e.g. plasma to dialysis buffer samples, and buffer to dialysis plasma samples) prior to protein precipitation and addition of internal standard. Each extract was then vortexed for 30 s and centrifuged at 17000 \times g for 10 min. A 1050 μ L aliquot of supernatant was evaporated to dryness using a Speed-Vac concentrator (Savant Instruments Inc., Farmingdale, NY,

USA). The residue was resuspended in 100 μ L of 95% v/v water (containing 0.1% v/v formic acid) and 5% v/v ACN, mixed, and centrifuged at 17000 x g for 10 min for further sample cleanup. The second supernatant was injected into the chromatographic system.

Method validation

Methods used for determination of D01-4582, celecoxib, mycophenolic acid and meloxicam were validated according to the Guidelines for Bio-analytical Method Validation published by the Food and Drug Administration in May 2001. Quantification of all analytes was performed using internal standard calibration. All QC samples and calibration standards were prepared in triplicate in canine plasma (for celecoxib, mycophenolic acid and meloxicam) or supernatant (for D01-4582). The lower limit of quantitation (LLOQ), detection (LLOD) and the concentration ranges used for calibration curves and QC for each analyte are listed in Table 2. Calibration curves were obtained with weighting of 1/x.

For all 4 analytes evaluated, the procedure produced a linear curve ($r^2 > 0.998$) over the concentration ranges evaluated with a high degree of repeatability. Both intra-day and inter-day precision CV were $< 15\%$, with accuracy (%Er) between -11 and 14%. All analytes were evaluated with run times of less than 5 min. All analytes were stable under the experimental conditions used in this study, with reproducible results (93 to 100% of the nominal concentration) after 24 hours at room temperature. Experimentally determined concentration values in plasma samples were considered acceptable with precision and accuracy higher than 85%.

Canine albumin gene sequencing

The canine albumin cDNA sequence has been reported for 26 Beagle dogs, identifying two linked SNPs at an allele frequency of 40% (Ito *et al.*, 2009). Since that initial report included only one dog breed, we sequenced all 14 exons of the canine albumin gene using genomic DNA from 100 dogs consisting of 37 breeds. DNA samples were obtained from the Washington State University College of Veterinary Medicine DNA Bank. By sequencing a wider variety of breeds, we intended to identify previously unreported polymorphisms in the canine albumin gene.

Primers were designed to amplify all 14 canine albumin exons from genomic DNA. The NCBI (www.ncbi.nlm.nih.gov) genomic DNA reference sequence NW_003726083 was used to design oligonucleotide primers and develop sequencing strategies for this study. The mRNA reference sequence (AB090854) was used for comparison. Primers were designed to anneal in the flanking intron DNA sequences to amplify each exon using PrimerQuest online design tool (<http://www.idtdna.com/Primerquest>). Standard PCR amplifications were carried out using 20 ng genomic DNA.

PCR products were visualized on a 1.2% agarose gel before treating the products with ExoSAP-IT reagent (Affymetrix Cleveland, OH, USA) according to manufacturer's directions. Treated PCR products were Sanger dye terminator sequenced with the same primers used for amplification using Big Dye 3.1 sequencing mix as directed (Applied Biosystems/Life Technologies, Grand Island, NY, USA). Sequence data were analyzed using Sequencher 5.2 software (GeneCodes Corp, Ann Arbor, MI, USA). Nucleotide sequences were evaluated for differences from the canine reference genome (CanFam3.1).

Canine albumin c.1075G>T and c.1422A>T genotyping

Canine albumin genotypes at two previously reported polymorphic sites (c.1075G>T and c.1422A>T) were determined using genomic DNA obtained from the WSU College of Veterinary Medicine DNA bank. DNA samples were randomly selected to include 1446 dogs representing 61 different breeds (minimum 10 dogs per breed) and 136 mixed-breed dogs. The breed of dog was based on owner designation at the time of admission to the WSU Veterinary Teaching Hospital. Genotyping was conducted using Taqman allele discrimination assays (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA) according to the manufacturer's directions. For c.1075G>T the primer/probes were 5'-CAA GTA AAA CTA TTT CAT TTT CAT CTG AAT CAG GT-3' (forward primer), 5'-TGG CGA GTC TCA AAA GCA ATG A-3' (reverse primer), 5'-TTG TAT GAA TAC GCA AGA AG-3' (G-allele probe labelled with Vic), and 5'-TTT TGT ATG AAT ACI CAA GAA G-3' (T-allele probe labelled with Fam). For c.1422A>T the primer/probes were 5'-GGC ACC AAA TGT TGT AAG AAA CCT-3' (forward primer), 5'-GTT AGC TTT GTC CAT TTC TAA AGG CAA A-3' (reverse primer), 5'-TGT GCT GAA GAC TTT-3' (A-allele probe labelled with Vic), and 5'-CTG TGC TGA IGA CTT T-3' (T-allele probe labelled with Fam). For the dogs included in the study, genotype results were confirmed with two separate runs.

Statistical analysis

All experimental measurements regarding the extent of plasma protein binding are reported as mean \pm SD, n = 12, unless otherwise noted. *In vitro* experiments were performed with 2 (D012-4582) or 3 (celecoxib, mycophenolic acid and meloxicam) replicates per sample, randomly distributed in each run, on three separate days. Descriptive and comparative analysis were done using Microsoft Office Professional Plus

Excel 2013 (Microsoft Corporation, Redmond, WA, USA) and SigmaPlot 12 software (Systat, San Jose, CA, USA) to analyze and report study results. ChemDraw 18.1 software (Cambridge, MA, USA) was used to construct Figure 1. Prism 3.03 GraphPad software (La Jolla, CA, USA) was used to construct Figure 3. Normality of the unbound concentrations for each analyte was assessed with Shapiro-Wilk test. Statistical power analysis was performed for sample size estimation, based on data from Ito et al. (2009), comparing dogs with and dogs without c.1075G>T and c.1422A>T variants. Median unbound fractions for all drugs were compared using Mann-Whitney U test to determine differences in the extent of plasma protein binding between dogs with haplotypes H1 and H2. Statistical difference between mean serum albumin concentration of each group was evaluated with unpaired t-test. For all comparisons, statistical significance was set at ≤ 0.05 .

RESULTS

Canine albumin exon sequencing

All 14 exons in the canine albumin gene were sequenced using DNA samples from 100 dogs representing 37 different breeds, and the results compared to the reference sequence (NW_003726083). Six exons (2, 6, 7, 10, 12 and 14) showed no variation from the reference sequence, while eight exons (1, 3, 4, 5, 8, 9, 11, and 13) contained SNPs. Of the SNPs identified, 13 were synonymous (data not shown) and 3 were nonsynonymous (Table 3). Among the 3 nonsynonymous SNPs, 1 was relatively uncommon, being only found as heterozygotes in 2 dogs (Irish Setter and American Eskimo Dog) out of 100 dogs tested. On the other hand, c.1075G>T and c.1422A>T were

present in 90 of 100 dogs sequenced. Due to the uncommon nonsynonymous SNPs' low frequency, they were not investigated further.

Effect of H1 and H2 allelic variation on drug binding to plasma

Drug plasma protein binding was evaluated in plasma samples collected from dogs homozygous for H1 allele (n = 6) and H2 allele (n = 6). Plasma albumin concentrations in all dogs tested (n = 12) was considered within normal limits (range: 2.5 to 3.3 mg/dL), with no statistically significant difference identified between H1 and H2 allele groups ($P = 0.41$). The extent of drug binding (expressed as percent bound) for all 12 dogs was 99.93 ± 0.04 (mean \pm SD) for D01-4582, 98.22 ± 0.3 for celecoxib, 95.36 ± 1.1 for mycophenolic acid and 94.90 ± 1.72 for meloxicam.

Considering the high extent of protein binding of the compounds analyzed as well as the pharmacological importance of unbound (free) drug, free drug fractions for each genotype are reported (Figure 2). Unbound fraction values calculated for each plasma-drug combination had a CV <20% (data not shown). Median unbound drug fractions with D01-4582 and celecoxib were considerably smaller (less than 2%) than those determined for mycophenolic acid and meloxicam. In plasma from dogs with albumin H1 allele, free drug fractions ranged from 0.02% to 0.13% (mean \pm SD; $0.06\% \pm 0.04\%$,) for D01-4582, 1.72% to 2.43% ($1.86\% \pm 0.34\%$) for celecoxib, 4.03% to 7.80% ($6.10\% \pm 1.29\%$) for meloxicam and 3.92% to 6.51% ($5.17\% \pm 0.83\%$) for mycophenolic acid. Plasma from dogs with albumin H2 allele had free drug fractions between 0.04% and 0.18% ($0.09\% \pm 0.05\%$) for D01-4582, 1.41% to 2.00% ($1.69\% \pm 0.19\%$) for celecoxib, 2.38% to 5.93% ($4.10\% \pm 1.55\%$) for meloxicam and 2.75% to 5.35% ($4.10\% \pm 1.11\%$) for mycophenolic

acid. For meloxicam only, the difference in free drug fraction between albumin genotypes reached statistical significance ($P = 0.0411$).

Canine albumin c.1075G>T and c.1422A>T haplotypes and breed distribution

c.1075G>T and c.1422A>T genotypes were determined in 1260 dogs from 61 different breeds and 186 mixed-breed dogs. Haplotypes that were resolved by inference are shown in Table 4 and haplotype frequencies for each breed are given in Supplemental Table 1. As shown in Figure 3, the frequency distribution of albumin haplotypes varied greatly across dog breeds. H1 (reference) and H2 (variant for both SNPs) were the predominant haplotypes in all dog breeds examined. Haplotype H1 was most frequently detected in Cocker Spaniels (95%), Miniature Dachshunds (89.5%) and Italian Greyhounds (75.6%), while haplotype H2 was most common in Great Danes (100%), Basenjis (98%), Rottweilers (97.5%), Golden Retrievers (97.1%), Boxers (95%), and Yorkshire Terriers (95%). Median (interquartile range; IQR) frequencies for haplotypes H1 and H2 across all breeds were 39% (25 - 50%) and 60% (50 - 71%), respectively. By comparison, H1 and H2 haplotype frequencies in mixed-breed dogs were 37% and 63%, respectively. Haplotype H3 (c.1075G>T) was found in 9 breeds, including Cirneco dell'Etna, Pomeranian, Portuguese Podengo, Weimaraner, Pembroke Welsh Corgi, Australian Shepherd, Rhodesian Ridgeback, Peruvian Inca Orchid, and Afghan, with a median (IQR) breed frequency of 5.9% (2.8 - 14%). Haplotype H4 (c.1422A>T) was found in 11 breeds, including Afghan, Border Collie, Siberian Husky, Borzoi, Pharaoh Hound, Greyhound, Longhaired whippet, Whippet, Italian Greyhound, Silken Windhound, and Scottish Deerhound, with a median (IQR) breed frequency of 1.8% (1.1 - 4.0%). Afghan

was the only breed with all four haplotypes detected. Haplotypes H3 and H4 were not found in any of the 186 mixed-breed dogs tested.

DISCUSSION

In our study, canine albumin genotype affected plasma protein binding of meloxicam, with no significant difference detected between mean free fractions of albumin H1 (reference) and H2 (c.1075G>T and c.1422A>T) allele dogs for the other three drugs assessed (D01-4582, celecoxib and mycophenolic acid). Drugs selected for our study had high preferential binding to albumin sites I or II, and a narrow therapeutic index (Er et al., 2013). Meloxicam and mycophenolic acid were used in our experiments due to their preferential binding in humans to albumin site I and site II, respectively (Er et al., 2013; Shaw and Nowak, 1995). In addition, celecoxib and D01-4582 were used because of their reported pharmacokinetic variability in dogs that could be associated with differences in protein binding (Paulson et al., 1999a)(Ito et al., 2009).

Our results were consistent with mean extent of plasma protein binding previously reported for dogs (99.9% for D01-4582, 97% for meloxicam, 98.5% for celecoxib, and 95-97% for mycophenolic acid) (Ito et al., 2009; Busch et al., 1988; Paulson et al., 1999b; Morassi et al., 2018). For D01-4582, our findings differ from a previous report (Ito et al., 2009), where free drug fractions were greater in plasma from Beagles with albumin H2 allele than in plasma from Beagles with H1 allele. Free fractions of D01-4582 in Beagles were reported to be $0.029 \pm 0.017\%$ (CV = 59%, n = 35), with albumin H1 allele dogs having statistically significant lower values ($0.015 \pm 0.003\%$) when compared to albumin H2 allele dogs ($0.059 \pm 0.016\%$) (Ito et al., 2009). In our study population, D01-4582 free

fractions were somewhat higher (mean \pm SD, 0.07% \pm 0.04%), with no statistically significant difference between dogs with albumin H1 allele and albumin H2 allele. There are some possible explanations for the contrasting results between the two studies. First, there are differences in the canine populations assessed. Whereas a single research Beagle colony was evaluated in the previously published study, our experiments included plasma from multiple dog breeds. Lower free fractions of D01-4582 may be common in Beagles, however, the inclusion of different breeds, representing greater genetic heterogeneity, likely increased the group biological variability and resulted in higher free drug fractions. Evaluating plasma protein binding in dogs from multiple breeds likely provides a more comprehensive and representative assessment of the overall canine population, however, the higher variability in free drug fractions may have contributed to the lack of statistical differences between groups. A second explanation is associated with the potential variability of lipid fraction in the plasma from our study population. D01-4582 is a highly lipophilic drug with plasma protein binding > 99%, which makes it likely to bind to lipoproteins in the plasma. Ultracentrifugation leads to the formation of a superficial chylomicron layer that can make the collection of supernatant difficult. In plasma samples, the presence and size of this chylomicron layer can lead to overestimation of free drug fraction. Even though plasma samples were collected while fasting, they were obtained from dogs from a variety of breeds, food regimen and body condition scores that likely contributed to a lipid profile more diverse than the one obtained from the research Beagle colony used by Ito et al. (2009). Breed, as well as diet and body condition score have been identified as a source of difference in plasma lipids in dogs and could have contributed to higher biological variability in plasma lipid content in our

samples compared to colony research animals (Downs et al., 1993; Jeusette et al., 2004). For celecoxib, our findings suggest that albumin genotype is unlikely to have caused the pharmacokinetic variability reported by Paulson et al. (1999a).

Multiple factors other than genotype can affect binding to albumin in plasma and may have contributed to the differences noted between dogs. Endogenous compounds in plasma (e.g. globulins, cells or metabolites) can influence free drug fractions by competitively displacing drugs from albumin binding sites and/or inducing conformational changes in the protein (Otagiri, 2005). For example, bilirubin and free fatty acids can competitively bind to human albumin and increase free drug fractions of the antibiotic cefazolin *in vitro* (Decroix, et al., 1988). While the content of other plasma components in study samples was not controlled, we estimate their impact to be small for celecoxib, meloxicam and celecoxib considering their high binding preference for albumin at the drug concentrations used in our experiments (below reported C_{max} in dogs). Lower albumin concentrations can also increase drug free fractions, in particular for drugs highly-bound to albumin (Ikenoue et al., 2000). Plasma albumin concentrations in our study were considered normal and unlikely to impact our results, with no statistical difference in albumin concentrations between allele groups. Albumin can also undergo post-translational modifications (e.g. glycation, cysteinylolation and carbamoylation) that can cause structural changes to the molecule and affect albumin's ability to interact with some drugs (Lee and Wu, 2015). These were not assessed in our study population. Sex-related differences in our plasma protein binding experiments were not statistically significant ($P > 0.05$).

Albumin genotype seemed to impact plasma protein binding of meloxicam in our *ex vivo* study, as dogs with albumin H1 allele had significantly higher free drug fraction than dogs with albumin H2 allele ($P = 0.041$). This contrasts the initial hypothesis that albumin H1 allele would be associated with lower free drug fractions (and therefore higher binding). Unlike the other 3 drugs tested in our study, meloxicam is the only one that binds to albumin site I in humans (Er et al., 2013). While preferential binding site for these drugs has not been extensively evaluated in canine albumin, it is possible that the role of albumin genotype in plasma protein binding is drug-dependent. While the amino acid changes associated with albumin H2 allele (c.1075G>T and c.1422A>T) may decrease binding of drugs commonly bound to site II, they may have the opposite effect on drugs bound to a different region of the albumin molecule in dogs. The difference in mean free drug fraction of meloxicam between albumin genotypes noted *ex vivo* may be relevant and should be confirmed *in vivo*. While changes in free drug fraction can affect total drug concentrations *in vivo*, the impact on free drug concentrations and pharmacologic effect is usually minor.

Another new finding of our study is that c.1075G>T and c.1422A>T polymorphisms are independent, occurring separately in a small percentage of dogs. While median allele frequencies in Beagles in our study population were similar to those in a previous report (Ito et al., 2009), they varied considerably between dog breeds. This difference in allele frequencies may be particularly important for veterinary drug development programs since single-breed studies may introduce bias in preclinical results.

Interestingly, the presence of more than one form of canine albumin was first reported in 1985, with two albumin phenotypes (“slow” and “fast”) identified based on their

electrophoretic mobility in a large population of dogs (Christensen *et al.*, 1985). The H2 allele breed frequencies from our study had a strong correlation ($R_s = 0.86$; $P < 0.0001$) with the corresponding frequencies for the slow (S) form reported by Christensen *et al.* (1985) (Figure 4). This indirectly suggests that the slow migration pattern of the S variant could result from the two predicted amino acid changes associated with the presence of the H2 allele. Unfortunately, we were unable to reproduce the starch gel electrophoresis technique used in the previous report to determine the electrophoretic mobility (phenotype) of the dogs used in our study.

The findings of our study should be interpreted considering its limitations. Comparing binding properties between genetic variants has previously been done using a small number of marker ligands for different binding regions of human serum albumin (Kragh-Hansen U *et al.*, 1990; Vestberg *et al.*, 1992). While binding properties of human albumin have been extensively investigated, similar information regarding canine albumin is scarce. Canine albumin is expected to follow the same tertiary molecular structure as human albumin, however, it may differ in the number and specificity of binding sites (Kosa *et al.*, 1997). Therefore, it is possible that the drugs we intended to interrogate sites I and II may actually bind to different sites on canine albumin. Our findings cannot predict the impact of c.1075G>T and c.1422A>T polymorphisms on specific binding sites and/or on plasma protein binding of other compounds. For that reason, additional studies evaluating the impact of albumin genotype on the extent of plasma protein binding of a large variety of drugs are recommended. While the difference in plasma protein binding of meloxicam seen in our *ex vivo* study suggests a significant impact of albumin genotype on free drug fraction, this should be confirmed with *in vivo* studies. The lack of significant differences

in free drug fractions of D01-4582, celecoxib and mycophenolic acid between albumin genotypes in our study is likely unrelated to the number of dogs evaluated in each group (n = 6) since the previous report (Ito et al., 2009) included only five dogs with H2 allele that showed statistically higher free drug fractions of D01-4582, when compared to H1 allele dogs.

In conclusion, this study provides novel information on the frequency and pharmacokinetic impact of the most common nonsynonymous canine albumin polymorphisms (c.1075G>T and c.1422A>T) across multiple breeds. In our study, dogs with albumin H1 allele had significantly higher free drug fractions (and consequently lower plasma protein binding) of meloxicam than dogs with albumin H2 allele. Albumin alleles H1 and H2 seem to be the most frequent across breeds and c.1075G>T and c.1422A>T are not completely linked, a finding that has not been previously reported. Our findings suggest that albumin genotype may influence free drug fractions at physiologically relevant concentrations of select drugs in dogs. Because changes in plasma protein binding commonly do not affect free drug concentrations, the significance of the influence of albumin genotype *in vivo* will depend on the drug studied and its intrinsic clearance. However, albumin genotyping may improve the interpretation of pharmacokinetic data generated in canine research populations during the developmental process of human and veterinary drugs.

ACKNOWLEDGMENTS

This research received funding from Wisdom Health, Mars Petcare (Franklin, TN) and the Program in Individualized Medicine, Washington State University (Pullman, WA).

Dr. Court was supported by the US National Institutes of Health grant [R01-GM-102130] and the William R. Jones endowment to Washington State University College of Veterinary Medicine. We thank Dr. Jane Wardrop and Dr. Leigh Knodler at Washington State University for providing the canine plasma used for method development and access to the ultracentrifuge, respectively. We thank Dr. Ito, at Daiichi Sankyo Co., Ltd., for providing a sample of D01-4582 for our study, as well as Dr. Sol Rivera and Darren Schnider for technical assistance.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Villarino, Court, Mealey, Costa.

Conducted experiments: Costa, Burke, Zhu.

Contributed new reagents or analytic tools: Mealey, Court, Villarino.

Performed data analysis: Costa, Court, Villarino, Mealey.

Wrote or contributed to the writing of the manuscript: Costa, Villarino, Court, Mealey.

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FOOTNOTES

- ^a Dr. Costa was supported by Wisdom Health, Mars Petcare (Franklin, TN) and the Program in Individualized Medicine, Washington State University (Pullman, WA). Dr. Court was supported by the US National Institutes of Health grant [R01-GM-102130]
- ^b description of protein sequence variants used by the authors is based on recommendations by the Human Genome Variation Society, with the position of the amino acid substituted calculated based on the precursor peptide (proalbumin) and not the mature peptide as in the paper by Ito et al. (2009).
- ^c SEKURE Chemistry Albumin assay, (Sekisui Diagnostics, Lexington, MA).

LEGENDS FOR FIGURES

Figure 1. Chemical structure for D01-4582, meloxicam, celecoxib and mycophenolic acid.

Figure 2. Scatter dot plots of unbound fractions of D01-4582 (A), celecoxib (B), meloxicam (C) and mycophenolic acid (D), in relation to albumin haplotype (H1 and H2) for each dog evaluated (n = 6 per group; average of 3 replicates from 3 separate runs). Lines represent median unbound fraction for each compound. The difference between median unbound fraction for both haplotypes was statistically significant for meloxicam (P = 0.041) but no other of the compounds analyzed (Mann-Whitney U test, significance set at P <0.05*).

Figure 3. Albumin H1, H2, H3 and H4 allele frequencies determined for 61 dog breeds and mixed-breed dogs. In parentheses are the number (n) of dogs sampled per breed.

Figure 4. Comparison of H2 haplotype frequencies determined in this study with frequencies of the albumin electrophoretic mobility slow (S) form allele reported previously by Christensen *et al.*, (1985) for the same dog breeds. Also shown is the Spearman correlation coefficient and associated P-value (significance set at P <0.05).

TABLES

TABLE 1. Relevance of the drugs selected for our protein binding experiments in dogs.

| Drug | Clinical relevance in dogs | Drug class clinically relevant in dogs | Presumed binding to albumin site I | Presumed binding to albumin site II | Previously studied in dogs |
|----------------------|----------------------------------|---|---|--|----------------------------------|
| D01-4582 | | N/A ^a | | x | x ^b |
| Meloxicam | x | nonsteroidal anti- inflammatory drug | x | | |
| Celecoxib | | nonsteroidal anti- inflammatory drug | | x | |
| Mycophenolic acid | x | Immunomodulatory | | x | |

^a Previously reported; ^b Ito et al., 2009

TABLE 2. Concentrations used to validate the methods in the quantification of D01-4582, meloxicam, mycophenolic acid and celecoxib in canine plasma.

| | LOD (μM) | LLOQ (μM) | QC (μM) | Concentration range (μM) |
|-------------------|--------------------------|---------------------------|----------------------|--|
| D01-4582 | 0.002 | 0.01 | 0.014, 0.045, 0.11 | 0.01 - 0.12 |
| Meloxicam | 0.02 | 0.03 | 0.09, 1.2, 2 | 0.045 - 2.2 |
| Mycophenolic acid | 0.01 | 0.025 | 0.078, 7, 16 | 0.03 - 14 |
| Celecoxib | 0.03 | 0.05 | 0.15, 0.8, 4.4 | 0.06 - 4.5 |

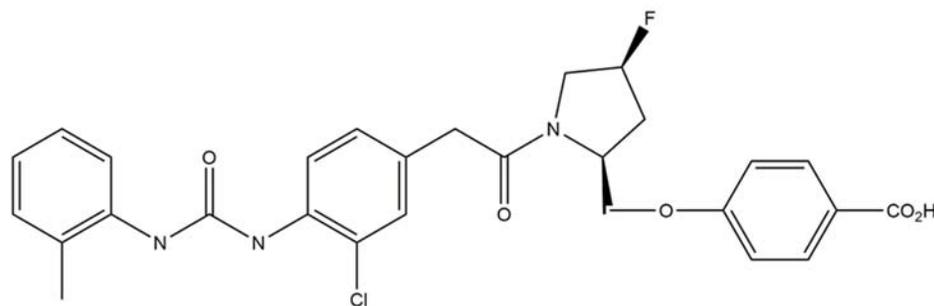
TABLE 3. Canine albumin gene nonsynonymous SNPs identified by sequencing all exons in 100 dogs representing 37 different breeds. The position of each variant is given in reference to the first nucleotide of the coding sequence (for cDNA) or first amino acid of the predicted protein. Listed are the numbers of dogs (n) with the reference (REF), heterozygous (HET), or homozygous variant (VAR) sequence for each SNP.

| Exon | cDNA | Amino acid | Number of dogs (n) | | |
|------|-----------|-------------|--------------------|-----|-----|
| | | | REF | HET | VAR |
| 3 | c.268C>T | p.Leu91Phe | 98 | 2 | 0 |
| 9 | c.1075G>T | p.Ala359Ser | 24 | 32 | 44 |
| 11 | c.1422A>T | p.Glu474Asp | 24 | 30 | 46 |

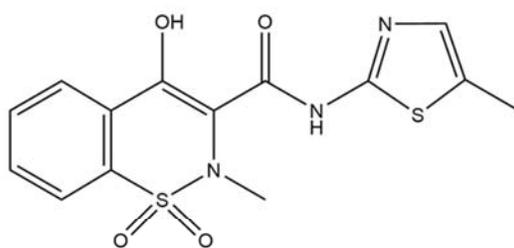
TABLE 4. Canine albumin c.1075G>T and c.1422A>T haplotypes.

| Haplotype | c.1075G>T | c.1422A>T |
|-----------|-----------|-----------|
| H1 | G | A |
| H2 | T | T |
| H3 | T | A |
| H4 | G | T |

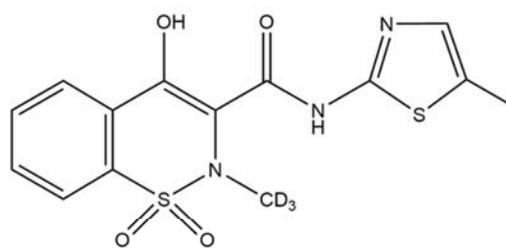
FIGURES



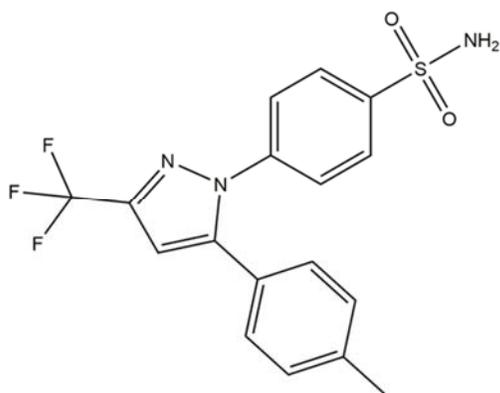
D01-4582



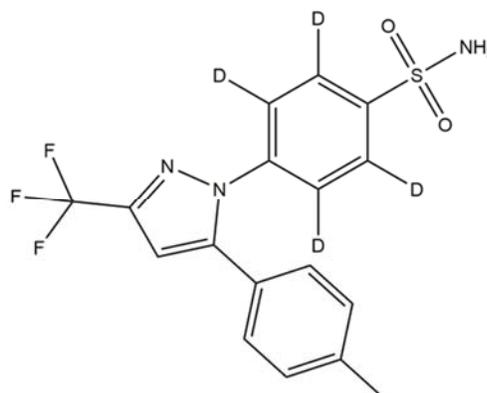
Meloxicam



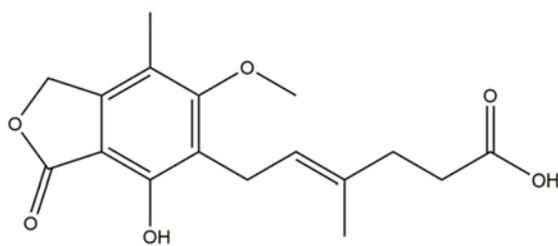
Meloxicam-d3



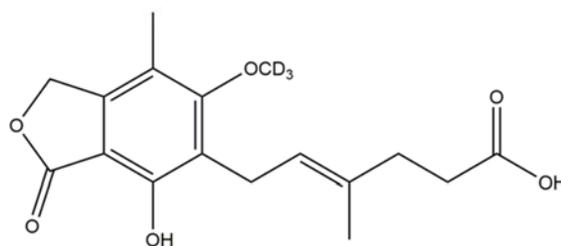
Celecoxib



Celecoxib-d4



Mycophenolic Acid



Mycophenolic Acid-d3

Figure 1.

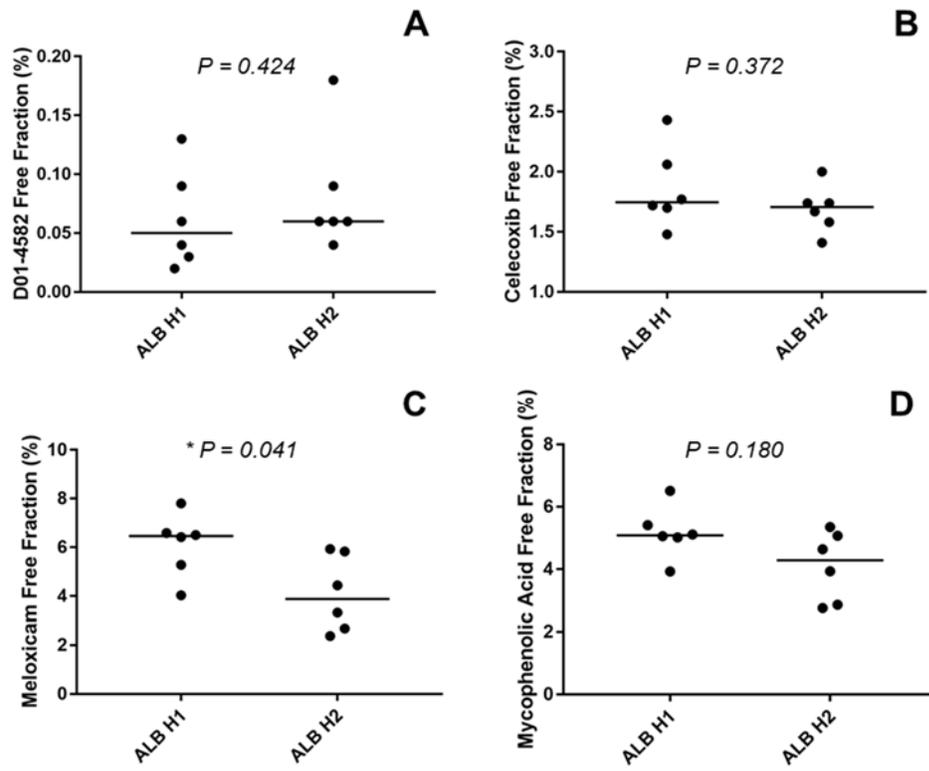


Figure 2.

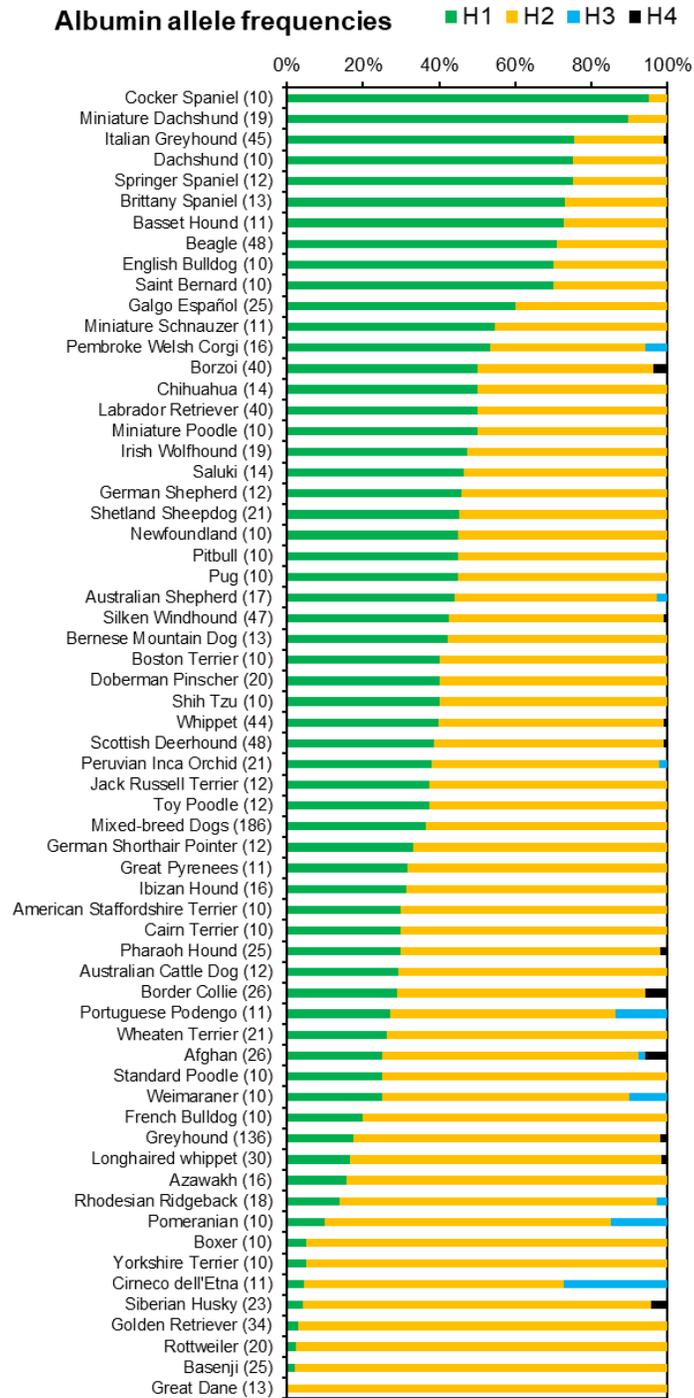


Figure 3.

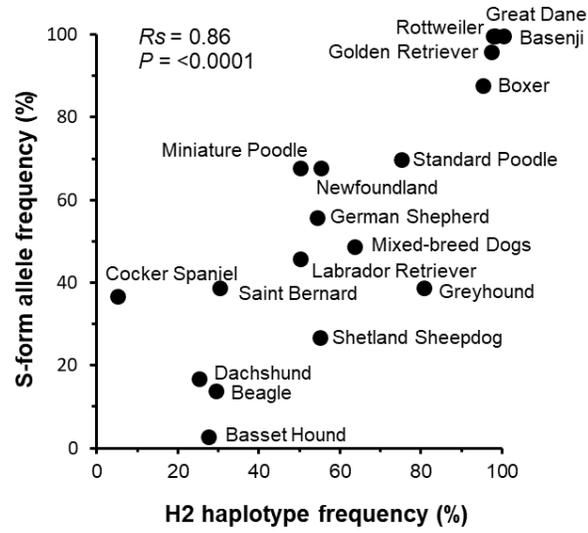


Figure 4.

TABLE 1. Canine albumin gene haplotype frequencies according to breed.

| | n | HAPLOTYPE FREQUENCY (%) | | | |
|-----------------------------------|----|-------------------------|------|-----|-----|
| | | H1 | H2 | H3 | H4 |
| Afghan | 26 | 25 | 67.3 | 1.9 | 5.8 |
| American Staffordshire Terrier | 10 | 30 | 70 | 0 | 0 |
| Australian Cattle Dog | 12 | 29.2 | 70.8 | 0 | 0 |
| Australian Shepherd | 17 | 44.1 | 52.9 | 2.9 | 0 |
| Azawakh | 16 | 15.6 | 84.4 | 0 | 0 |
| Basenji | 25 | 2 | 98 | 0 | 0 |
| Basset Hound | 11 | 72.7 | 27.3 | 0 | 0 |
| Beagle | 48 | 70.8 | 29.2 | 0 | 0 |
| Bernese Mountain Dog | 13 | 42.3 | 57.7 | 0 | 0 |
| Border Collie | 26 | 28.8 | 65.4 | 0 | 5.8 |
| Borzoi | 40 | 50 | 46.3 | 0 | 3.8 |
| Boston Terrier | 10 | 40 | 60 | 0 | 0 |
| Boxer | 10 | 5 | 95 | 0 | 0 |
| Brittany Spaniel | 13 | 73.1 | 26.9 | 0 | 0 |
| Cairn Terrier | 10 | 30 | 70 | 0 | 0 |

Drug Metabolism and Disposition # 87304

| | | | | | |
|--------------------------|-----|------|------|------|-----|
| Chihuahua | 14 | 50 | 50 | 0 | 0 |
| Cirneco del'Etna | 11 | 4.5 | 68.2 | 27.3 | 0 |
| Cocker Spaniel | 10 | 95 | 5 | 0 | 0 |
| Dachshund | 10 | 75 | 25 | 0 | 0 |
| Doberman Pinscher | 20 | 40 | 60 | 0 | 0 |
| English Bulldog | 10 | 70 | 30 | 0 | 0 |
| French Bulldog | 10 | 20 | 80 | 0 | 0 |
| Galgo Español | 25 | 60 | 40 | 0 | 0 |
| German Shepherd | 12 | 45.8 | 54.2 | 0 | 0 |
| German Shorthair Pointer | 12 | 33.3 | 66.7 | 0 | 0 |
| Golden Retriever | 34 | 2.9 | 97.1 | 0 | 0 |
| Great Dane | 13 | 0 | 100 | 0 | 0 |
| Great Pyrenees | 11 | 31.8 | 68.2 | 0 | 0 |
| Greyhound-NGA | 136 | 17.6 | 80.5 | 0 | 1.8 |
| Ibizan Hound | 16 | 31.3 | 68.8 | 0 | 0 |
| Irish Wolfhound | 19 | 47.4 | 52.6 | 0 | 0 |
| Italian Greyhound | 45 | 75.6 | 23.3 | 0 | 1.1 |
| Jack Russell Terrier | 12 | 37.5 | 62.5 | 0 | 0 |
| Labrador Retriever | 40 | 50 | 50 | 0 | 0 |

Drug Metabolism and Disposition # 87304

| | | | | | |
|----------------------|-----|------|------|------|-----|
| Longhaired Whippet | 30 | 16.7 | 81.7 | 0 | 1.7 |
| Miniature Dachshund | 19 | 89.5 | 10.5 | 0 | 0 |
| Miniature Poodle | 10 | 50 | 50 | 0 | 0 |
| Miniature Schnauzer | 11 | 54.5 | 45.5 | 0 | 0 |
| Mixed-breed | 186 | 36.6 | 63.4 | 0 | 0 |
| Newfoundland | 10 | 45 | 55 | 0 | 0 |
| Pembroke Welsh Corgi | 16 | 53.5 | 40.6 | 5.9 | 0 |
| Peruvian Inca Orchid | 21 | 38.1 | 59.5 | 2.4 | 0 |
| Pharaoh Hound | 25 | 30 | 68 | 0 | 2 |
| Pitbull | 10 | 45 | 55 | 0 | 0 |
| Pomeranian | 10 | 10 | 75 | 15 | 0 |
| Portuguese Podengo | 11 | 27.3 | 59.1 | 13.6 | 0 |
| Pug | 10 | 45 | 55 | 0 | 0 |
| Rhodesian Ridgeback | 18 | 13.9 | 83.3 | 2.8 | 0 |
| Rottweiler | 20 | 2.5 | 97.5 | 0 | 0 |
| Saint Bernard | 10 | 70 | 30 | 0 | 0 |
| Saluki | 14 | 46.4 | 53.6 | 0 | 0 |
| Scottish Deerhound | 48 | 38.5 | 60.4 | 0 | 1 |
| Shetland Sheepdog | 21 | 45.2 | 54.8 | 0 | 0 |

Drug Metabolism and Disposition # 87304

| | | | | | |
|-------------------|----|------|------|----|-----|
| Shih Tzu | 10 | 40 | 60 | 0 | 0 |
| Siberian Husky | 23 | 4.3 | 91.3 | 0 | 4.3 |
| Silken Wind hound | 47 | 42.6 | 56.4 | 0 | 1.1 |
| Springer Spaniel | 12 | 75 | 25 | 0 | 0 |
| Standard Poodle | 10 | 25 | 75 | 0 | 0 |
| Toy Poodle | 12 | 37.5 | 62.5 | 0 | 0 |
| Weimaraner | 10 | 25 | 65 | 10 | 0 |
| Wheaten Terrier | 21 | 26.2 | 73.8 | 0 | 0 |
| Whippet | 44 | 39.8 | 59.1 | 0 | 1.1 |
| Yorkshire Terrier | 10 | 5 | 95 | 0 | 0 |
