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FACULDADE DE MEDICINA VETERINÁRIA

DETECTION OF THE MDR1 MUTATION IN PORTUGUESE DOG BREEDS

MARIA CRISTINA TENREIRO PEREIRA RODRIGUES BARROSO

ORIENTADOR(A):
Doutora Berta Maria Fernandes Ferreira São Braz

COORIENTADOR(A):
Dr. Gonçalo Eduardo Vítor Vicente

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Abstract

DETECTION OF THE MDR1 MUTATION IN PORTUGUESE DOG BREEDS

P-glycoprotein is an ATP-driven drug efflux carrier, encoded by the multidrug resistance gene MDR1, also been referred as ABCB1, that is responsible for the transport of a broad variety of compounds, including drugs commonly used in veterinary medicine, out of the cell against the concentration gradient. The influence of P-gp on drug disposition has been demonstrated in Collies and in other herding dog breeds since a severe intoxication in response to treatment with the antiparasitic drug ivermectin and other avermectins has been reported in a subpopulation of these breeds. This adverse reaction is related to a 4-bp deletion in the ABCB1 gene.

To our knowledge, no study was conducted in portuguese dog breeds to detect this gene mutation and there is no available information for the clinicians about this fact and consequently, about the safety of the administration of drugs that are P-gp substrates. Thus, it is important to know the status about the presence of MDR1 in dog breeds in Portugal.

The main objective of this project was to implement the genetic test to identify the gene mutation on MDR1 gene and to perform this analysis in several animals from dog breeds in Portugal to obtain their MDR1 genotype. For that, we performed biological samples of saliva in animals from the dog breeds belonging to Group 1 and from the ones already identified as affected. The diagnosis technique used was adapted from the ones utilized by other authors, namely Mealy and collaborators.

We analyzed 105 animals, 21.9% of which are Barbado da Terceira, 9.5% are Cão da Serra d'Aires, 52.4% belonging to breeds known to carry the mutation and 16.2% to other breeds.

With this study we were able to establish the analysis in our laboratory, we identified the mutation in dogs of breeds already signalized as having the mutation and we evidenced that the mutation already is in Barbado da Terceira - carriers.

Keywords: MDR1/ABCB1, P-glycoprotein (P-gp), portuguese dog breeds, herding breeds, gene mutation, phenotype, genotype, P glycoprotein substrates.

Resumo

DETEÇÃO DA MUTAÇÃO MDR1 NAS RAÇAS CANINAS PORTUGUESAS

A glicoproteína P é um transportador dependente de ATP, codificado pelo gene de resistência a fármacos MDR1, também conhecido como ABCB1, que é responsável pelo transporte contra o gradiente de concentração (para o espaço extracelular) de vários substratos, incluindo fármacos comummente utlizados em Medicina Veterinária. A influencia deste transportador na reação a fármacos foi demonstrada em Collies e outras raças pastoras devido ao desenvolvimento de sinais neurológicos, de intoxicação grave, após o tratamento destes animais com antiparasitários do grupo das avermectinas, nomeadamente, a ivermectina. Esta reação está relacionada com a deleção de 4 pares de base no gene canino ABCB1, descoberta em 2001, em cães com fenótipo sensível à ivermectina.

Até à data, não temos conhecimento de nenhum estudo feito em Portugal para detetar esta mutação genética e não existe informação disponível para os clínicos sobre este facto e, consequentemente, sobre a segurança de administração de medicamentos que sejam substratos da gp-P. É, por isso, importante saber-se o estatuto MDR1 em raças caninas em Portugal.

O principal objetivo deste trabalho foi implementar o teste genético para identificação da mutação genética no gene MDR1 e realizar esta análise em vários exemplares de raças caninas em Portugal para obter o seu genótipo MDR1. Para o efeito foram realizadas colheitas de amostras biológicas de saliva em exemplares das raças caninas pertencentes ao grupo I e das raças já identificadas como afetadas. A técnica de diagnóstico utilizada foi adaptada das técnicas utilizadas por outros autores, nomeadamente Mealey e colaboradores.

Foram analisados 105 animais, 21.9% dos quais são Barbados da Terceira, 9.5% são Cão da Serra d'Aires, 52.4% pertence a raças já identificadas como portadoras da mutação e 16.2% a outras raças.

Com a realização deste estudo, conseguimos estabelecer a técnica no nosso laboratório, identificámos a mutação em cães de raças já sinalizadas como tendo a mutação e detetámos evidências que a mutação também circula na raça Barbado da Terceira - portadores.

Palavras-chave: MDR1/ABCB1, glicoproteína P (gp-P), raças caninas portuguesas, raças pastoras, mutação genética, fenótipo, genótipo, substratos da glicoproteína P.

List of abbreviations and symbols

ABCB1 ATP-binding cassette 1

ABCG2 ATP-binding cassette G2

ADME Absorption, Distribution, Metabolism and Excretion

ADR Averse Drug Reaction

BBB Blood Brain Barrier

ACE Angiotensin Converting Enzyme

ADRB1 β_1-adrenergic receptors

BCRP Breast Cancer Resistance Protein

CEBEA Comissão de Ética e Bem Estar Animal

cGMP Cyclic Guanosine Monophosphate

CNS Central Nervous System

CPC Clube Português de Canicultura

CPR Cardiopulmonary Resuscitation

CPVL Centre de Pharmacovigilance Vétérinaire de Lyon

CSF Cerebrospinal Fluid

CT Computerized Tomography

CYP3A Cytochrome P450 3A

DNA Deoxyribonucleic Acid

EMA European Medicines Agency

FCI *Federation Cynologique Internationale*

GABA Gamma Amino Butyric Acid

GI Gastrointestinal

HEV – FMV Hospital Escolar Veterinário – Faculdade de Medicina Veterinária

MA Marketing Authorization

MDR1 Multidrug Resistance 1

PDE5A Phosphodiesterase 5A

P-GP P-glycoprotein

RNA Ribonucleic Acid

SLC Solute Carrier Superfamily

SNP’s Single Nucleotide Polymorphism

UGT UDP-glucuronyl transferase

UIDI Unidade de Isolamento para Doenças Infecto-Contagiosas

VMP Veterinary Medicinal Product

WHO World Health Organization

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I. Activities during the internship and the externship

The curricular internship, during the 6th year of the Mestrado Integrado em Medicina Veterinária, took place in HEV – FMV – Universidade de Lisboa, under the supervision of Prof. Dr. Berta Maria Fernandes Ferreira São Braz and of Dr. Gonçalo Eduardo Vítor Vicente. A schedule was developed by the latter, coordinator of the HE-FMV curricular internships, combining not only an 8 hours period per day but also day and night shifts, with 12 hours duration, sometimes these days being at the weekend. In the end, the internship lasted for 26 weeks, with a total of 1120 hours.

The beginning of the internship was in Primary Care, under the surveillance of the veterinarians, nurses, and technicians. Here the student have the opportunity to assist and initiate first time and second opinion appointments, regarding different areas, including: emergency, vaccination, deworming, gastrointestinal (GI) related problems, kidneys, skin, heart, endocrine, respiratory diseases among other. The student was frequently responsible for initiating the appointments and should collect all the history, clinical signs and symptoms of the animals and do the physical exam. After this procedure, the assistant veterinarian assumes the consultation. The student should discuss the diagnostic exams and the best treatment for each case. She also discussed the medications and prepared other ones to be administered in the hospital. This way, the student had the opportunity to increase some important capacities such as the communication with animal owners, how to analyze the clinical signs and symptoms and how to link them with the patient's frame, to develop the clinical reasoning. Alongside with these inquiries, the student also took part in several procedures such as: patients' restraint, blood and urine collection among other sampling that were needed for diagnostic tests.

During the Primary Care context, the student assisted the nurses doing dressings, blood sampling, drug administration, animal restraint and collection of other biological samples At the emergency and hospitalization environment, the student had to do crucial tasks such as catheter placement, calculation and preparation of fluid therapy systems, preparation and administration of drugs intravenously, intramuscularly, subcutaneously and orally, placement of urinary catheters, tracheal intubation, drainage of pleura and/or of the abdominal cavity, cardiopulmonary resuscitation (CPR), supervision of critical patients and monitoring inpatients twice a day, dressings and colocation of patches.

Following this first internship part, the student spent 80 hours on Dermatology and Allergology Service, under the guidance of Dr. Hugo Pereira and Prof. Dr. Mafalda Lourenço. Here, attended to reference consultations, first-time visits and review appointments. Besides collecting the anamneis, the student could do some specific procedures such as cutaneous

and auricular cytology, Wood lamp test, skin biopsies, cytology by the tape technique, hair sampling for mycological analysis and microscopic visualization, intradermic tests, and so on. The therapy and control methods for the skin and fur affections were discussed as well.

The permanence on the Unidade de Isolamento para Doenças Infecto-Contagiosas (UIDI) lasted for one week, with a total of 40 hours, beneath the guidance of Prof. Dr. Solange Gil, Dr. Inês Machado and Dr. Eva Cunha. This unit is an isolated confinement for patients with infectious diseases (suspected or confirmed) or when the health status is unknown (intermediate care). Over here, the student had contact with hygiene and security procedures and with equipment for individual safety. Regarding medical procedures, they are the same already described for general medicine and internment.

The student spent 4 weeks (160 hours) on the Small Animal Surgery Service, except for the emergency surgeries, out of working hours. This rotation took place under the orientation of Dr. Leonor Iglésias, Dr. Ana Reisinho, Dr. António Martinho, Prof. Dr. António Ferreira, Prof. Dr. Lisa Mestrinho, together with the nursing team. Here the student had contact with several types of surgery including soft tissues, orthopedic, neurologic and dentistry, working as a surgeon's assistant, anesthetist and circulating person. The daily rotation includes the animal's reception and pre-surgical preparation (intravenous catheterization, pre-medication), followed by the anesthetic induction with a fixed anesthetic, intravenous fluid therapy administration, endotracheal intubation and maintenance of the anesthesia with a volatile anesthetic, trichotomy, washing and disinfection as well as correct positioning of the animal. As surgeon's assistant, the student helped the surgeon in any case needed, gaining important expertise about the techniques that should be used in certain cases and, finally, had the opportunity to practice suture techniques. As an anesthetist, the animals were monitored with regard to the anesthetic plan by regulating the anesthetics outflow. After each intervention, the student did the post-surgical monitorization of the patients with the performance of bandages, administration of medication, extubation and discharge notes to the tutors.

On Radiology Services, the student spent 120 hours between computerized tomography (CT) and Radiography beneath the orientation of Prof. Dr. Sandra Jesus, Dr. Óscar Gamboa and Dr. António Almeida. Here, she applied the anesthetic techniques already mentioned, developed her interpretation capacity for radiographic studies and CT images, as well as how to use the equipment for individual safety.

The student stayed for 80 hours in the Internal Medicine Service, under the guidance of Prof. Dr. Rodolfo Leal. Here, she assisted the appointments, discussed the cases and saw the performance of high endoscopy and rhinoscopy, both to preform biopsies. In this service, were addressed pathologies of the endocrine, gastrointestinal, nephrological, immunological, hematological and respiratory systems.

During the 80 hours on Ophthalmology, with Prof. Dr. Esmeralda Delgado and Dr. Ana Marta Amorim, the student attended consultations and surgeries, as well as specific diagnostic methods such as gonioscopy, Schirmer test, measurement of intraocular pressure, direct and indirect ophthalmoscopy, fluorescein test, etc.

After, she did an 80 hours rotation on Oncology Service, with Dr. Gonçalo Vicente and nurse Élia Cosme. Over here the student had contact with preparation (dose calculation, protection measures and preparation place) and administration of chemotherapy, using protective equipment. Every chemotherapy day started with the reception of the animals, blood collection for complete blood count and catheter placement. During the other days, the student attended oncology appointments.

The last 80 hours of the internship were spent on the Ultrasound Service under the orientation of Dr. Rui Lemos Ferreira and Dr. Ana Isabel Filipe. Here she was able to assist to abdominal, scrotal and cardiac ultrasound studies. The student learned how to identify organs in the studies, assist to centesis such as cystocentesis (some of those were performed by her), abdominocentesis and thoracocentesis, as well as biopsies. Like in the other services, she discussed the cases with the presentation of differential diagnoses and the best approach in each case.

At the end of the internship, the student was able to develop communications skills, clinical and critical thinking, technical and performance capacity.

After the internship the student went to Texas A&M University Small Animal Veterinary Medical Teaching Hospital and spent 80 hours in the Ultrasound Service, learning how to perform an ultrasound study besides the other things already mentioned in the Ultrasound Service in HEV-FMV, under guidance of Dr. Andra Vogues, Dr. Jim Griffin, Dr. Kathy Spaulding, Dr. Brian Poteet, residents Dr. Alex Ohlendorf, Dr. Michelle Acierno, D. Ashley Yanchik and Dr. Gwen Levine. In the Cardiology Service, she spent 80 hours attending to consultations, seeing echocardiograph studies, as well as electrocardiograph studies. She learned how to place an Holter, identify murmurs and its classification and how to approach some cardiac emergencies, abnormalities and diseases. In this service, she shadowed Dr. Sonya Wesselowski and residents Dr. Katrina Cusak and Dr. Blakeley Janacek.

II. The MDR1 mutation on Portuguese herding dog breeds – State of the art

1. Introduction

In 1976, Juliano & Ling, identified a deletion of 4bp in the gene MDR1, leading to a series of stop codons, in a Chinese hamster ovary cell line, selected in culture for resistance to colchicine. This deletion would give the cells the ability to resist to chemotherapeutic agents. Later, in 2001, Mealey and her collaborators, identified the same deletion in cells of mice and dogs that experienced toxic reactions after the administration of ivermectin.

The product of this gene is P-glycoprotein (P-gp), that is a 170-kD transmembrane protein pump and exists in the apical membrane of brain capillary endothelial cells as well as in other organs such as small intestine, kidney and placenta, functioning as a way to extrude the substrates from the endothelial cell to the capillary lumen (Roepe 1995). Its substrates include large, structurally unrelated hydrophobic compounds such as digoxin, cyclosporin and ivermectin (West 1990 Feb). The malfunction of this protein results, for example and with specific significance, in the failure of the blood-brain-barrier which leads to the accumulation of its substrates in the brain tissue with neurologic signs.

Ivermectin, as well as other avermectins, are widely used in veterinary medicine as antiparasitic drugs due to their board spectrum of action (nematode and arthropod parasites). In the parasite, avermectins act potentiating glutamate-gated chloride channels and gamma amino butyric acid (GABA)-gated chloride channels of the peripheral nervous system (Jansson and Dybas 1998). In most mammals, these compounds are not able to access the brain as result of the blood-brain-barrier (BBB), and since mammalian GABA receptors are exclusively located in the central nervous system (CNS) they are generally protected from the effects of avermectins. Additionally, there are some transporters like P-gp that extract substrates from the brain tissue, preventing their accumulation and toxicity.

There are some specific groups of genetically engineered mice and some specific dog breeds that are extremely sensitive to the neurological action of avermectin due to the deletion on MDR1 gene. These mice proved to be 50-100 times more sensitive to ivermectin related neurotoxicity than the wild-type mice (Schinkel et al. 1994) and accumulated 90-fold higher concentrations of the compound in the brain than do wild-type mice.

The breeds that are most affected by the mutation are herding breeds belonging to group 1 breeds (according to the FCI - Federation Cynologique Internationale) such as Collie, Longhaired Whippet and Australian Shepperd among others. Since that, to our knowledge, no study was made in Portuguese dog breeds that belong to this group, or any other one, we

decided to test Portuguese dogs to detect the mutation, warning the veterinary clinicians if it is present in the populations and the dog owners, so they can reproduce their dogs consciously.

2. Pharmacogenetics vs Pharmacogenomics

The concept that gene expression could explain individual variation in drug susceptibility and/or drug efficacy, was first proposed in 1957 and the term pharmacogenetics appeared two years later. However, this field remained little explored until the initiation of the Human Genome Project in the '90s. From that moment, pharmacogenetic research has increased at an outstanding rhythm (Mealey, 2018).

Pharmacogenetics studies the variability in drug response, due to heredity (Pirmohamed, 2001) generally, used to describe a little number of genes involved on drug metabolization (Court 2007), whereas pharmacogenomics encompasses all the genes involved in the genome that can interfere with drug response, focusing on the “express genome” and techniques to study drug responses (Ginsburg and Willard 2016). Genetic variations can affect the pharmacodynamics (interaction with receptors and transporters) and the pharmacokinetics [absorption, distribution, metabolism, and excretion (ADME)] of a specific drug (Mealey, 2018).

With the progress of molecular techniques, current pharmacogenetic investigations are slightly different from the initial ones. Right now, it includes the identification of the phenotype and the genetic variation responsible for it. Nowadays, the researchers are focused on identifying functionally important variations in deoxyribonucleotide acid (DNA) sequences in genes that have a significant impact on drug response and, most of the times, mutations are identified before the phenotypic consequence is known. When significant alterations in drug response are identified in pharmacogenetic studies, a patient’s overall reactions to a particular drug require pharmacogenomic researches to characterize the individual contribution of multiple genes to drug response (Mealey, 2018). However, this distinction is arbitrary, and both terms are interchangeable, and the ultimate achievement is personalized medicine, leading to more efficacy and safety regard drug therapy. Personalized medicine pertains to combined expertise regarding the personal susceptibility to a certain disease, prognosis and response to therapy, thus enhance a individual's health (Swen et al. 2007).

The access to the genome of the veterinary species has speed up the development of pharmacogenetic discoveries, making easier the ultimate goal of an individualized drug therapy (Mealey, 2018). The studies of these areas are in their very beginning and research opportunities are emerging, since the molecular level until the clinical one, that are covering fields as diverse as pharmacology, toxicology, molecular biology, internal medicine,

epidemiology, etc., (Pirmohamed 2001). Probably, they are the ultimate way to establish the best treatment (what drug and dose) for each patient, minimizing adverse reactions and optimizing the efficacy (Mealey, 2018).

It is important to highlight that the individualization of the therapy comprises two significant clinical implications. First is the capacity to anticipate those patients at high risk of developing drug toxicity, patients who might have a mutation that affects a drug-metabolizing enzyme, decreasing the clearance. This implies alteration on dosage, dosing interval or in the prescribed drug. Second, is the ability to prescribe a specific drug to a specific patient based on its drug receptors rather than using a trial-and-error method because patients with mutations on drug receptors may present a different response to particular medication (Mealey, 2018).

2.1. Implications of pharmacogenetics in the absorption of orally administered drugs

Systemic bioavailability of an oral drug depends on a physicochemical characteristic of the drug and its hepatic metabolism. However, several other factors influence the capacity of a drug to be absorbed into the bloodstream after oral administration. One of the most important determinants of bioavailability of orally administered drugs is the intestinal phase I drug metabolism by, for example, cytochrome P450 3A (CYP3A) and the drug efflux by, for example, P-glycoprotein (P-gp) (Mealey, 2018). Drug molecules can be degraded by luminal, cytosolic, brush border oxidative and other hydrolytic and conjugative enzymes that are a barrier to drug absorption and posterior bioavailability (Patel and Mitra 2001). Thus, genetic variation in intestinal drug metabolization that leads to abnormal enzymes and abnormal transporters as well as alterations on hepatic enzymes and transporters will affect oral drug absorption (Mealey, 2018).

Drug transporters such P-gp are also known to play a significant role in drug absorption and bioavailability. P-gp is a stark example of the potential impact of a drug transporter and the pharmacogenetics on molecule pharmacokinetics (Schinkel et al. 1995), as will be further elaborated. Other drug transporters that are known to affect the bioavailability of orally administered drugs are breast cancer resistance protein (BCRP), encoded by the ABCG2 gene, in mice and humans with functional polymorphism, but the substrate drugs for this transporter still unknown (Mealey, 2018).

2.2. Pharmacogenetics affecting drug distribution

The systemic distribution of a drug can be drastically affected by pharmacogenetics. One of the best examples of that is P-gp that is a great barrier to drugs to achieve certain tissues selecting if drugs can or cannot cross the blood-brain-barrier (BBB), the blood-testis-barrier and the placenta. Thus, the distribution of P-gp substrates, for example, ivermectin and loperamide, to these tissues is increased in dogs with MDR1 deletion mutation, as will be explained below. Less information is available concerning P-gp and the BBB in cats, but there are studies in development (Mealey, 2018).

Another important drug transporter that also plays a significant role in drug distribution is ABCG2 or BCRP that functions as a drug efflux pump in a variety of tissues, including liver, intestine, BBB, blood-retina barrier and mammary gland (Lindner et al. 2013). In tumoral tissues, ABCG2 avoids the intracellular accumulation of cytotoxic drugs, conferring multidrug resistance to the cancer cell. However, some polymorphisms of this transporter result in abnormal drug disposition, increasing sensitivity to adverse drug reactions (Mealey, 2012). This transporter was first discovered in humans when the common therapy was administered and failed, and has been associated with some diseases such as gout (Martinez, Court, Fink-Gremmels, & Mealey, 2018). The mammary gland is the main engine of the excretion of both therapeutic and toxic compounds toward milk affecting the concentration of substrate drugs, such as fluoroquinolones, in milk. For this reason, polymorphism of BCRP has important implications in drug residues in milk and mastitis therapy (Lindner et al. 2013). Furthermore, cats have several amino acid differences that greatly affect transport function relative to human ABCG2. Last of all, some rodent studies indicate that ABCG2 may have a relevant role in regulating some physiologic pathways involving protect erythrocytes from oxidative damage (Mealey, 2012).

2.3. Implications of pharmacogenetics in drug metabolism

The biotransformation of xenobiotics in the body is divided into two phases: I and II. Phase I reactions transform the primary compound to a more polar substance(s) by exposing or *de novo* formation of functional groups. These reactions include dealkylation, aliphatic and aromatic hydroxylation, oxidation and deamination. The principal enzymes involved in the process are cytochromes P450 enzymes performing hydroxylations and acting as mono and dioxygenases and hydrolases. These enzymes are heme enzymes, and they are responsible for the metabolism of xeno and endobiotics and are also involved in a variety of biosynthetic processes. Phase II enzymes biotransform the xenobiotics and the endogenous compounds

in molecules that are more easily excreted as well as inactivated pharmacologically active substances. These enzymes are mostly transferases and carry out conjugating reactions including glucuronidation, sulfation, methylation, acetylation, glutathione and amino acid conjugation (Jancova et al. 2010). Currently, most of the knowledge regarding pharmacogenetics in humans is related to genetic variations in drug metabolizing enzymes and is known that can affect both Phase I and Phase II metabolic enzyme activity (Mealey 2018).

The drug-metabolizing enzymes of CYP450 are essential to an efficient elimination of many drugs, but in humans and, probably, in all veterinary species, exists a considerable interindividual variability in the activity of these enzymes. Thus, for a specific drug dosage, the resulting effect can range from sub-therapeutic to toxic. These variations can result from exposition to CYP enzyme inhibitors or inducers in diet, from the interaction between coadministered medications or from genetic variation (Court 2013a).

In veterinary species, only a few polymorphisms in drug metabolizing enzymes have been described until now. The most well-described polymorphisms are from canine CYPs, especially a mutation in the coding region of CYP1A2 that generates a premature stop codon so the homozygous affected dogs lack hepatic CYP1A2 protein expression and enzymatic function (Tenmizu et al. 2004; Mise et al. 2008). The known substrates for CYP1A2 in dogs are caffeine, phenacetin and theophylline (Court 2013a). When looking for the polymorphism in Beagles in Japan, investigators detected that 11-17% of the dogs were homozygous for the mutant genotype, thus lacking functional CYP1A2. In these dogs, a concentration of the tested drugs was 17 times greater than in normal dogs. However, in the heterozygous dogs, the plasma concentrations were not significantly different from the wild-type dogs' concentrations. Nevertheless, the implications of this polymorphism in the pharmacokinetics, efficacy and safety of the clinically used drugs are unclear and depend on: the presence of CYP1A2 in the general population and the dependence of the drug on CYP1A2 for clearance (Tenmizu et al. 2004; Mise et al. 2008). The breeds with the greatest allele frequency are Irish Wolfhound, Beagles (mostly japanese ones, in Europe and United Stated the allele frequency is lower) and Berger Blanc Suisse. A large number of the other breeds that were found to exhibit the mutation have allele frequencies lower than 10% but were the same herding breeds known to have MDR1 mutation (Court 2013a).

Another phase I drug-metabolizing enzyme with phenotypical variation in dogs is CYP2B11. This enzyme is known to be involved in anesthetic drug hypersensitivity and affects mostly Greyhounds and other sighthound breeds (Court 2013a). It has been proposed that these dogs have a prolongated recovery after utilization of injectable anesthetics agents such as thiopental, thiamylal and propofol (Mealey 2018). It was believed that this delay was due to decreased drug redistribution from the central compartment because of the reduced body fat

in Greyhound. However, later studies attributed the effect to reduced drug clearance when compared to mix-breed dogs and when compared with dogs with the previous administration of microsomal enzyme inducer – phenobarbital. Other studies demonstrated that propofol clearance was also slowed in Greyhounds and the preadministration of a CYP inhibitor (chloramphenicol), slowed, even more, the clearance of propofol. Despite that, there is no report of a molecular genetic basis for this breed-dependent variance (Court 2013a).

Regarding phase II metabolic enzymes, there are two panspecies defects that are relevant in veterinary medicine, one affecting cats (UDP-glucuronyl transferase – UGT) and the other affecting dogs (*N*-acetyltransferase) (Mealey 2018).

UGT defect in cats affects drastically drug disposition, however, these defects cannot be considered a true pharmacogenetic defect, because these defects are genetic variations among species, instead of within-species variations (Mealey 2018). Cats are deficient in several UGTs, but not all. They are specifically lacking in UGTs that are responsible for the metabolism of phenolic compounds so they cannot metabolize compounds like acetaminophen. This explains some of the idiosyncratic reactions that are reported in cats (Court 2013b).

Concerning dogs, they have another panspecies phase II metabolic-enzyme defect – lack of *N*-acetyltransferase (*NAT*). This enzyme is responsible for the metabolization of important drugs such as procainamide, hydralazine, sulfonamide antibiotics, isoniazid and other drugs. Both *NAT1* and *NAT2* genes are nonexistent in dogs, raising the risk of adverse reactions and hypersensitivity (Trepanier et al. 1997). Cats are deficient in *NAT2* but express *NAT1* (Court 2013b).

A phase II metabolic enzyme that has a true pharmacogenetic variation is thiopurine methyltransferase (TPMT) enzyme. This latter is responsible for the metabolization of azathioprine, a cytotoxic thiopurine antimetabolite drug and its active metabolites to the inactive forms. It appears that exists a nine-fold range in this enzyme activity and the activity level is dependent on the breed because Giant Schnauzers had lower TPMT activity whereas Alaskan Malamutes had increased activity. The lower TPMT activity is linked with increased susceptibility to azathioprine-induced bone marrow suppression (Kidd et al. 2004).

2.4. The influence of pharmacogenetics in drug excretion

Drugs can be eliminated from the body intact or as metabolites. The two most important excretion pathways are renal and biliary excretion; however, excretion may occur by other paths too. As an example, we have P-gp which, as will be explained later, has an important

impact on renal and hepatic excretion because it is expressed on renal tubular cells and biliary canalicular cells.

Another important superfamily of transporters is the solute carrier superfamily (SLC) consisting of both uptake and efflux transporters. This superfamily exists at least in humans and rodents, but probably also exists in other veterinary species. There is some intersection not only between substrates but also between these and the transporters in humans and rodents. SLC transporters exist over the body's barrier tissues such as intestine, liver, kidney, placenta, brain capillary endothelial cells among others. At these specific locations, SLC transporters can control drug absorption, distribution, metabolism and excretion. This superfamily includes multidrug and toxin extrusion transporters, organic cations (genetic variations are known in humans that affect the plasma concentration and clinical effects of ondansetron) and anions, as well as organic anion transporting peptides (Mealey 2018), however the author is not aware of genetic variants in this superfamily in the veterinarian species.

2.5. Pharmacogenetics of drug receptors

Recently, pharmacogenetic exploration reached the area that involves the identification of polymorphism in genes encoding drug receptors and actuating proteins. Some polymorphism has been identified in angiotensin converting enzyme (ACE), β-adrenergic receptors, dopamine, estrogen and other receptors, in humans. These polymorphisms can have severe implications in clinical outcomes: ACE polymorphism is associated with a higher risk of death or at least transplantation in patients with heart failure; polymorphism in β_1-adrenergic receptors (ADRB1) can either improve or inhibit effects of adenylyl cyclase activity, based on the type of the polymorphism (Mealey, 2018). The ADRB1 polymorphism has been identified in both dogs (Maran et al. 2013) and cats (Maran et al. 2012). In these last ones, three polymorphisms were identified within the ADRB1 gene. Two of these polymorphisms does not modify the amino acid so is unlikely to have clinical significance, but the other one was an AA/CC replacement and the coded amino acid change from proline to glutamine. Despite that, further research is needed to understand the clinical implications of this polymorphism (Maran et al. 2012). However, in dogs, two in-frame deletion mutations were find and the preliminary studies indicated that these dogs are less sensitive to the effects of the atenolol, a β-adrenergic antagonist (Maran et al. 2013).

Another important canine polymorphism is the phosphodiesterase 5A (PDE5A) gene that has interactions with vasodilators like nitric oxide and sildenafil. The concentration of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase in a dog presenting the

mutant genotype was lower when compared to the wild-type dogs. cGMP is the target of the phosphodiesterase inhibitors. Despite the conclusion that the plasma concentration of cGMP is lower in these dogs, there is no evidence that their response to the therapy is impaired (Stern et al. 2014).

2.6. Rule of pharmacogenetics in the idiosyncratic reactions

Pharmacogenetic variations can affect not only the adverse drug reactions that are predictable and, usually concentration-dependent (type A reactions) but can also affect the idiosyncratic (type B) reactions as well. Although these reactions are called hypersensitivity, they not always involve an immunologic reaction and can be mediated by polymorphisms in the drug receptors like NAT2 (Mealey, 2018).

One of the drug classes that is commonly involved in idiosyncratic adverse reactions in both dogs and humans are the sulfonamides. The syndrome in dogs consists of fever, blood dyscrasias (neutropenia, thrombocytopenia, hemolytic anemia), arthropathy, skin eruptions, hepatopathy with cholestasis and necrosis, uveitis or keratoconjunctivitis sicca. Some dogs can also present protein-losing nephropathy, meningitis, pancreatitis, pneumonitis, or facial nerve paralysis (Trepanier, 2004). In humans, it has been shown that, slow acetylation by NAT2 is a risk factor for sulfonamide hypersensitivity reactions. It had been suggested that the alternative metabolic pathways in these individuals, generate reactive metabolites that establish covalent bindings to cell macromolecules leading to cytotoxicity and immune response to neoantigens. A specific genetic diversity has not been identified in dogs that experienced idiosyncratic reactions to sulfonamides, but the implication fo NAT2, CYB5A and CYP5R3, had been ruled out (Sacco et al. 2012).

2.7. Pharmacogenetics and protein binding

Xenobiotics can bind to several plasma proteins, with albumin being the most important one, for the drugs used in veterinary medicine. The fraction that is linked to the protein is not pharmacologically active, wherefore changes in protein binding may affect the toxicity and the efficacy of the drug that bound to these proteins. This linkage can also affect the pharmacokinetics depending on the degree of protein binding. Considering that only the free fraction of the drug is available for metabolism and/or excretion, the clearance of the drug can be either increased (if the unbound fraction increased) or decrease (if the unbound fraction decreased). The bound between protein and drug can be affected by several situations, including interactions between drugs and alterations in plasma albumin concentrations and the

pharmacogenetics itself may result in discrepancies regarding protein binding in different patients (Mealey, 2018).

3. Central Nervous System (CNS) – physiology

The entire CNS is covered by the meninges – *pia mater*, *arachnoid* and *dura mater* – all these layers have a protective role (Boron and Boulpaep 2012; Klein and Cunningham 2012).

The cerebrospinal fluid (CSF) is found in the subarachnoid space between the *pia mater* and the *arachnoid* and fills the central canal of the spinal cord and the cerebral ventricular system. Cerebrospinal fluid is primarily produced on cerebral ventricles (Abbott 2005) and is practically devoid of blood cells, contains a small amount of protein and shows differences in ion concentrations when compared to plasma (Klein and Cunningham 2012).

Since there are exchanges between the CSF and the extracellular fluid of the CNS, the CSF is a major determinant on the neuronal microenvironment, providing micronutrients to the cellular metabolism and draining metabolites resulting from it that, otherwise, could become toxic. CSF also has an important role in mechanic protection of the CNS, absorbing shocks that may occur (Klein and Cunningham 2012).

Regarding the BBB, it refers to the selective nature of the CNS vessels, concerning the substances that can or cannot cross the vessels, protecting the CNS against neurotoxic substances, which explains the difficulty to reach pharmacologically the brain and de spinal cord (Klein and Cunningham 2012).

3.1. The blood-brain barrier

The fact that some dyes, when put on circulation, dye some tissues but not brain tissue, suggested that there is a selective permeability in cerebral vessels, which is responsible for restricting some substances from coming into contact with the brain. This mechanism is called the BBB and it is responsible for the maintenance of the environment around the neurons and glial cells, in between the physiological limits. This protector barrier is necessary since the composition of blood can significantly vary with a very wide range of factors such as age, food supply, health condition, metabolic activity, exercise or exposure to environmental toxins, etc. (Daneman and Prat 2015). Many of the blood components (metabolites, toxins, nutrients) are neuroactive and have the ability to affect and modify the function of ionic channels, receptors and membrane transporters whereby the BBB avoid deregulation of them as well as avoid changes in neuronal function (Abbott 2005; Klein and Cunningham 2012).

In most capillary networks, the water-soluble solutes diffuse freely by concentration gradient between the cells that comprise the endothelium. In the cerebral capillary network, this solutes exchange is limited by tight junctions, being highly selective (Abbott 2005; Abbott et al. 2010). In general, non-protein-bound, liposoluble, uncharged small molecules (O_2, CO_2, etc.) will easily cross the cerebral capillary endothelium. The remaining types of molecules, such as glucose and some amino acids, only cross the cerebral capillary endothelium through mechanisms mediated by specific transporters. Furthermore, endothelial cells can express some degradative enzymes that restrict even more the substances that are able of cross the BBB (monoamine oxidase, for example). Thus, it is easy to understand that ischemic events or episodes of trauma can compromise the BBB integrity and its protective capacity (Klein and Cunningham 2012).

In contrast, sometimes these mechanisms can be an obstacle on therapies to be intended because they do not allow the majority of the antibiotics as well as other drugs with low molecular weight (few liposoluble drugs, bound to plasma proteins, etc.) to penetrate the BBB, so they do not reach the brain as it was needed. Moreover, some substances whose characteristics allow them to freely cross de BBB, are actively and rapidly withdrawn back into the bloodstream by active transport.

In brain regions involved in the control of serum osmolarity, glycemia, hormonal intercommunication, food and water intake, vomiting and control of the concentration of other solutes, BBB is not effective because it loses the tight junctions responsible for its function. These areas, such as the hypothalamus, are known as *circumventriculares* organs.

4. P-glycoprotein (P-gp)

The Chinese hamster ovary cell line, selected in culture for resistance to colchicine (Juliano and Ling 1976), expressed a large amount of a 170kD protein, later named P-glycoprotein, were resistant not only to colchicine but also to other amphiphilic drugs. During the 80's and the 90's several studies were done on this glycoprotein, since in tumor cells, conferred resistance to colchicine, resistance to vinblastine and doxorubicin (Ueda et al. 1987; Juranka et al. 1989) and has been found to exist in the luminal surface of healthy epithelial tissues such as adrenal glands, liver, kidneys, intestine and placenta (West 1990). There was an attempt to develop drugs able to inhibit P-gp function in the tumor cells, so that they return to their phenotype of response to chemotherapy (Mealey, 2008), which resulted in the identification of several molecules able to inhibit the efflux activity of P-gp, but without great success in terms of use, since many other mechanisms of cell resistance to chemotherapeutic agents were identified (Michieli et al. 1999).

Physiologically, the expression of P-gp has a very important role, mainly on the tissues with excretory and/or protective functions such as the brush border of enterocytes in the small intestine, in the canalicular membrane of hepatocytes (Figure 2), in the endothelial cells of the capillaries of the brain (Figure 1) and testes and in the brush border of proximal tubule cells in the kidney (Pauli-Magnus and Kroetz 2004), being a key point in the intestinal absorption of drugs administered orally, as well as in the biliary and urinary excretion and in the distribution of the molecules in specific barrier protected tissues as brain and testes (Pauli-Magnus and Kroetz 2004).

Its major function is to prevent the intracellular accumulation of xenobiotics and to control the exposure to potentially toxic compounds, considering that P-gp has two principal mechanisms of action: first, by alteration of the intracellular compartments, leading to exchanges in the electrical potential of the membrane and/or increasing the intracellular pH, resulting to a decrease in drug accumulation within the cell (Roepe, 1995). And second through the *pump model*, wherein the P-gp uses the energy released in the hydrolysis of ATP to remove molecules from the cell membrane and cytoplasm in analogy to the ion transport pumps (Gottesman et al. 1996), that is, the P-gp carries the molecules back to the lumen of the capillaries constituting the BBB, of the bile ducts and those of the renal tubules. As can be seen in Figure 1, in the normal dog, the radiolabeled substrate does not cross the BBB, so the brain tissues does not became radiolabeled, in contrast with the mutant dog in which the radiolabeled substrate crosses the BBB and accumulates in the brain tissue.

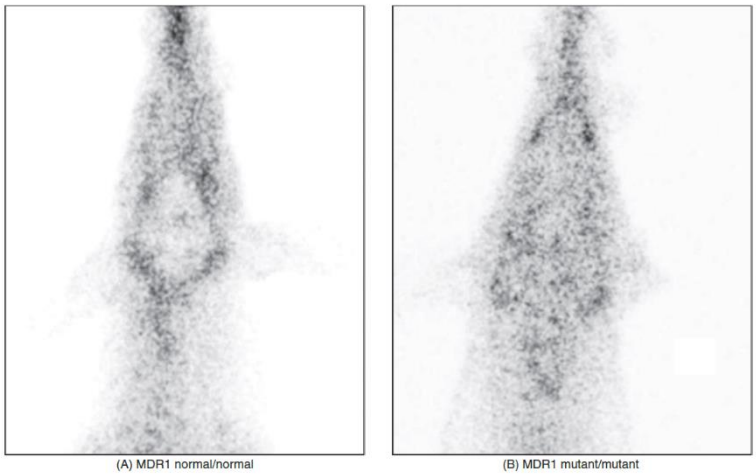

Figure 1 Nuclear scintigraphy imaging the head of two Collies (image reproduced from Mealey (2018), with permission from Wiley, Blackwell)

(A) MDR1 normal/normal dog. (B) MDR1 mutant/mutant dog. In the normal dog, the radiolabeled substrate does not cross the BBB, so the brain tissue is not labeled. In the mutant dog, the radiolabeled substrate is accumulated in the brain tissue.

Mammalian P-gp has broad substrate specificity, transporting molecules with diverse chemical structures, including chemotherapeutic agents (doxorubicin, vinca-alkaloids – vinblastine), immunosuppressants (cyclosporin and tacrolimus), macrocyclic lactones and

steroid hormones (Seelig 1998) and it turns out that most of these substrates have a natural origin or are derived from natural compounds, which reinforce the theory about its protective function against xenobiotics, however, the mechanism by which P-gp recognizes and transports such different molecules is still unknown. Most P-gp substrates are also cytochrome P450 3A4 substrates, so we can infer that P-gp plays a very important role in the effect and response *in vivo* to many drugs, and is also involved in numerous pharmacological interactions, most relevant when speaking of drugs with narrow therapeutic index, where induction or inhibition of the carrier may have very significant impacts on the efficacy and safety of the drug (Greiner et al. 1999; Dürr 2000)

4.1. Role of P-gp in intestinal absorption

P-gp is mainly expressed on the luminal surface of the small intestine, limiting the absorption of substrates transported by it. Drugs with absorption potentially limited by P-gp includes digoxin, dexamethasone, cyclosporin A, opioids, fluoroquinolones, beta-adrenergic antagonists (McEntee et al., 2003; Mealey, 2006). The lower influence on the absorption of orally administered molecules may also be associated with the saturation of the P-gp, since the molecule concentration in the intestinal lumen is very high and the capacity of transport is limited (Martinez et al., 2008a). Furthermore, it is known that the inhibition of P-gp increases the absorption of its substrates, such as cyclosporine A, HIV-1 Protease inhibitors (humans), beta-adrenergic antagonists, opioids, ivermectin, digoxin, dexamethasone, fluoroquinolones and other drugs (Kim et al., 1998; Kwei et al., 1999; Mealey, 2004; Schinkel et al., 1995; Schwarz et al., 1999; Seelig, 1998; Wandel, Kim, Wood, & Wood, 2002; Westphal, 2000; Yamaguchi, Yano, Saito, & Inui, 2002).

4.2. Role of P-gp in the kidney

Regarding the normal human kidney, the P-gp and the MDR1 RNA were identified in the proximal tubules, mesangial cells, the thin branch of the loop of Henle and the collecting ducts (Ernest and Bello-reuss 1997). However, the highest concentration is found on the apical surface (luminal face) of the proximal tubule cells, the target site of most nephrotoxic substances, and the region responsible for the elimination of potentially toxic compounds (Miller 2002).

P-gp serves as one of the major efflux transporters in the kidney and several cases of drug interaction have been attributed to the attenuation of active tubular secretion, such as the

inhibition of apical P-gp by quinidine or verapamil will decrease the excretion of the P-gp substrate digoxin (Sun, Frassetto, & Benet, 2006).

P-gp transports mainly neutral or cationic substrates and some polypeptides, what seems to have an important role in reducing renal toxicity of xenobiotics. This may explain why nontoxic doses of doxorubicin become toxic to mouse mesangial cells when co-administered with other P-gp substrates such as verapamil and cyclosporin (Muller and Jansen 1997).

4.3. P-gp role in biliary excretion

Bile is the result of active transport of its components into the biliary space and, primarily, is formed by the hepatocytes and, secondarily, is secreted at the bile canaliculus. The canalicular space and the intercellular spaces are separated for tight junctions creating two domains – apical and basolateral – that differ in their lipid composition and tight junctions, which are believed to provide effective barriers against lateral movement of lipids and proteins and allows the maintenance of different environments between bile and interstitium (Roberts et al. 2002).

The ATP-dependent export pumps in the canalicular membranes are members of the ATP-binding cassette family (Keppler and Arias 1997) and the P-gp is only expressed in this side of the membrane of the hepatocytes, which emphasizes its importance to mediate the efflux of xenobiotics and endogenous substances into the bile as a detoxification pathway (Kwon et al. 1996).

The results of the study of Coelho et al. (2009) support the hypothesis that P-gp is very important to biliary excretion because MDR1 mutant/mutant dogs have a significantly decrease biliary excretion of the substrate while MDR1 normal/normal dogs have a normal biliary excretion (Figure 2). Furthermore, MDR1 mutant/normal dogs presented an average phenotype. So, it can be concluded that blood and tissue concentrations of P-gp substrates are likely to reach higher levels that can be toxic and persist longer rising the risk of adverse reactions.

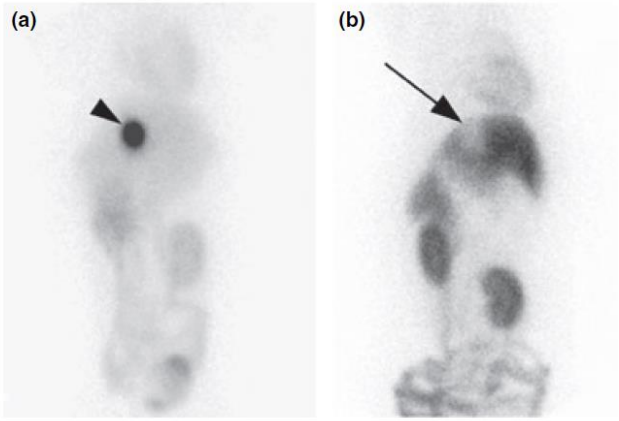

Figure 2 Liver scintigraphy. From Coelho et al. (2009) with permission of the Journal of Veterinary Pharmacology and Therapeutics

(a) MDR1 normal/normal dog. (b) MDR1 mutant/mutant dog. In the normal dog, the radiolabeled substrate is confined to the gallbladder and does not appear in the kidneys, while in the mutant dog both liver and kidney parenchyma appeared marked with the substrate.

4.4. P-gp and drug metabolism

Even known that P-gp does not appear to have an intrinsic metabolic function, it has a close relation with CYP 3A and had been showed that P-gp plays an important role in intestinal drug metabolism. When an orally administered drug reaches the intestine, it is passively absorbed into the enterocyte. Once there, three possibilities occurs: a) be metabolized by CYP 3A; b) go into the bloodstream; c) be pumped out into the intestine lumen again by P-gp, where it can enter in another enterocyte more distally along the GI tract and get access to CYP 3A being metabolized or extruded again. This will lead to the non-substrates of P-gp only passing once in the enterocyte whereas P-gp substrates may continually circulate among the enterocyte and the intestinal lumen, leading to repeated access of CYP 3A to the molecule or fecal excretion due to constant P-gp efflux. Because of their close interaction, it is difficult to establish the contribution of each protein to diminish oral drug absorption (Mealey, 2004).

4.5. P-gp modulation

According to Mealey (2004), all the changes in absorption, distribution, metabolism and excretion of P-gp substrates (Table 1) that have been substantiated in homozygous mice (mutant/mutant) are replicable in wildtype mice with the co-administration of a high-efficiency P-gp inhibitor (Table 2) when a P-gp substrate was administered. The same effect has been described in humans and dogs with the use of P-gp inhibitors in conjunction with P-gp substrates. These drug combinations are being studied in humans bearing in mind the

enhancing distribution of substrate drugs to the brain or testis or to increase the oral bioavailability of substrate drugs (Sun et al., 2004). In this way, the deficiency relative to P-gp can be a consequence of a genetic alteration in the MDR1 gene but can also occur due to pharmacological interactions and veterinarians may use it for the benefit of the patient.

4.6. P-gp and CYP 3A associated drugs

As previously mentioned, many drugs are at the same time substrates from P-gp (Table 1) and from CPY 3A which doses should be adjusted for heterozygous individuals and avoided in homozygous (mutant/mutant) individuals. Also, there are some drugs which can inhibit and influence P-gp and CYP 3A function (Table 2) that interferes in the absorption and excretion of these drugs when administered orally.

Table 1 P-gp substrates (from Mealey, 2016; Mealey, 2004)

Pharmacological group	Substances	
Macrocyclic lactones	Ivermectin Milbemycin Moxidectin	Selamectin Doramectin Eprinomectin
Octadepsipeptide	Emodepside	
Antimicrobial	Erythromycin* Ketoconazole Itraconazole* Tetracycline	Doxycycline Levofloxacin Sarafloxacin
Analgesic/Sedative	Acepromazine Butorphanol	
Chemotherapeutics	Actinomycin D Doxorubicin Vinblastine* Vincristine*	Vinorelbine Docetaxel* Paclitaxel
Opioids	Loperamide Morphine	
Antiemetic	Ondansetron	
Cardiac drugs	Digoxin Diltiazem*	Verapamil* Talinolol
Steroid hormones	Aldosterone Cortisol*	Dexamethasone* Methylprednisolone
Immunosuppressants	Cyclosporin* Tacrolimus*	
*Substrate of CYP 3A		

Table 2 P-gp inhibitors (Mealey 2004)

Pharmacological groups	Substances	
Antimicrobial agents	Erythromycin^{+} Itraconazole*	Ketoconazole^{+}
Opioids	Methadone Pentazocine	
Cardiac drugs	Verapamil* Amiodarone* Carvedilol	Quinidine* Nicardipine*
Antidepressants	Fluoxetine St John's wort	Paroxetine
Immunosuppressants	Cyclosporine* Tacrolimus*	
Miscellaneous	Bromocriptine Chlorpromazine	Tamoxifen* Grapefruit juice^{+}

*CYP 3A substrate

$^{+}$CYP 3 inhibitor

5. Basic genetic concepts

A gene is just the DNA sequence enclosing series of codons that specify proteins and the changes that occur among individuals in a population is a consequence of the sequence differences in specific genes that are the result of mutations. When a gene is expressed, DNA is transcribed to ribonucleotide acid (RNA), which is translated to proteins. A codon is formed by a three consecutive nucleotide base, that culminates in the addition of a particular amino acid or stop codons (signaling amino acid chain termination) but the genetic code is redundant, which means that the same amino acid is coded for two or more different codons. In dogs, for example, TAA, TGA and TAG represent stop codons (Mealey 2018).

Mutations change the sequence of nucleotide bases in a DNA molecule which leads to change in the transcribed RNA, creating different codons that can lead to stop codons or different amino acids that alter the structure and function of proteins after translation. However, these mutations can be silent due to the redundancy already described, and the base change can create a codon for the same amino acid as specified by the original DNA sequence with no change in the protein structure or function (Mealey, 2018).

Everyone has two alleles for each locus, one from every parent. The allele is the DNA sequence at a given gene's location on the chromosome and the individual can have a homozygous genotype if has two equal alleles or heterozygous genotype if has two different alleles. The phenotype of an individual concerning a single gene is the outcome, the physical manifestation of the genotype and this outcome can be easily observed like the color of the eyes or it can remain unapparent until, for example, a particular drug administration in an individual (Mealey, 2018).

6. The genetics of the dog

Canis familiaris, the domestic dog, with its 78 chromosomes, is the most phenotypically divergent mammal species, with nearly 400 modern breeds recognized and varying in size and conformation from a Shih Tzu to a Great Dane passing for Bulldogs, Yorkshire, Collies, Labradors, etc. (Ostrander 2012). There are some remarkable differences not only in the breed conformation, but also in behavior and physiology that reflect the intensity of artificial selection and, ultimately, the genetic variability of founding populations (Vila 1999). The DNA-DNA hybridization data revealed that the Canidae family dissented approximately 50 million years ago from other carnivore families. On the conversely, the current canids are very closely related and derivate from the same ancestor about 10 million years ago. Some studies demonstrate that individuals from the same breed share common alleles and can be grouped based on the population structure (Irion 2003; Koskinen 2003; Parker et al. 2004) and breeds that are used for the same task and that have similar morphology often share common alleles too (Parker et al. 2004; vonHoldt et al. 2010). Despite that, these studies do not take into account some important variables leading to the development of modern breeds, such as geographic separation and immigration, the hybridization and the timeline of the formation of breeds (Parker et al. 2017). These authors, overcome these limitations by presenting an extensive set of data, including pure breeds sampled from around the world and genotyped on a large scale. The authors applied the phylogenetic and genome-wide analysis of recent haplotype sharing (Figure 4), revealing an overlap between breed populations and so leading to the proposition of a two-step process for breed formation: an ancient separation based on the dog function and, more recently, selection based on physical attributes. All of this information allowed the authors to understand the linkage between mutations that are shared across breeds that seemed unrelated (Parker et al. 2017). They used samples from 127 different breeds and nine wild canids obtaining 150.067 informative single nucleotide polymorphism (SNPs). Selected to use distance measures based on allele sharing as a substitute for frequency and to improve the analyses with unprejudiced haplotype sharing for robust assessment of the population structure (Parker et al. 2017). They obtained a cladogram from 161 breeds (Figure 3). 91% (146) of these formed single and breed-specific nodes with 100% bootstrapsupport. Seven breeds (Belgian Tervuren, Belgian Sheepdog, Cane Corso, Bull Terrier, Miniature Bull Terrier, American Hairless Terrier and Rat Terrier) were part of two or three breed clades that were supported at 98% or more; Lhasa Apso and Saluki formed a single-breed clade supported at 50% and 78%. There were four breeds split within single multi-

breed clades (Jack Russell, Redbone Coonhound, Sloughi and Cane Paratore) and the last two breeds were split between divergent clades (Xoloitzcuintli and peruvian hairless dog).

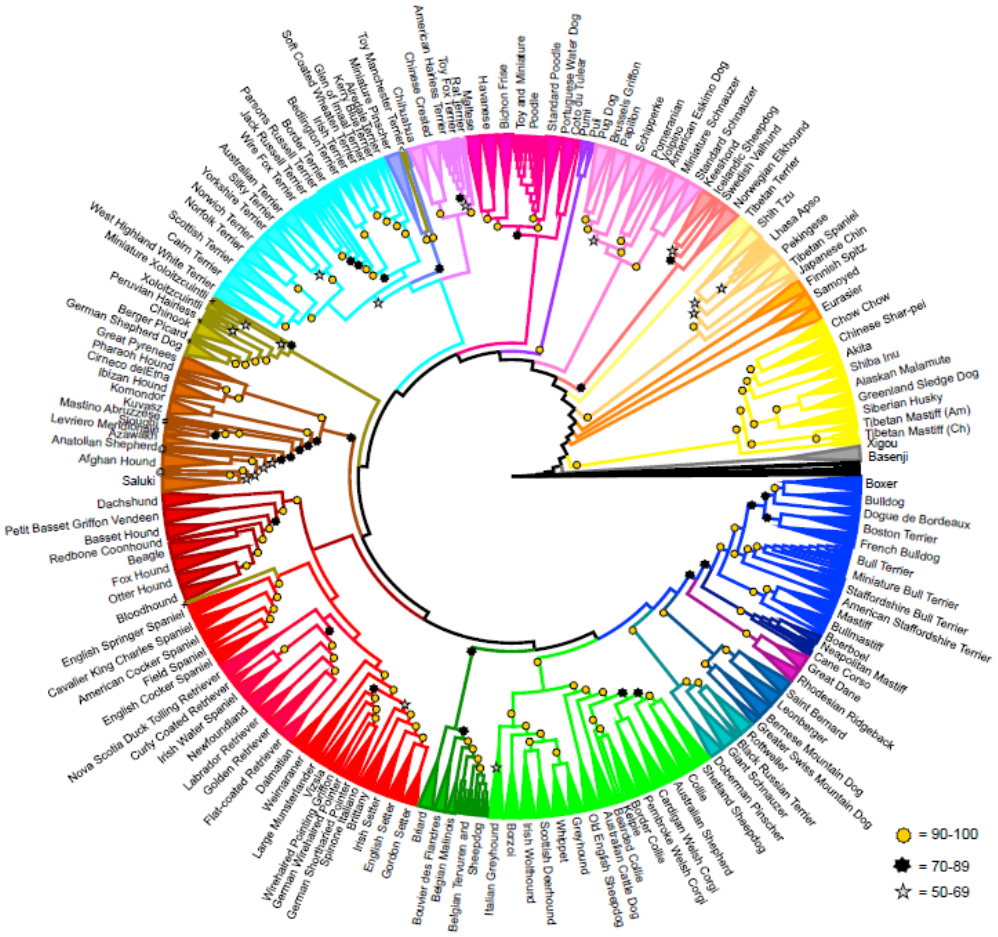

Figure 3 Cladogram of 161 domestic dog breeds

The breeds that form unique clades supported by 100% of bootstraps are combined into triangles. For all other branches, a gold star indicates 90% or better, black star 70%_89%, and silver star 50%_69% bootstrap support. Breeds are listed on the perimeter of the circle. A small number of dogs do not cluster with the rest of their breed, indicated as follows: *cane paratore, +Peruvian hairless dog, #sloughi, @country-of-origin salukis, and ^miniature xoloitzcuintle. Adapted from Parker et al., 2017 with permission from Elaine A. Ostrander, one of the authors.

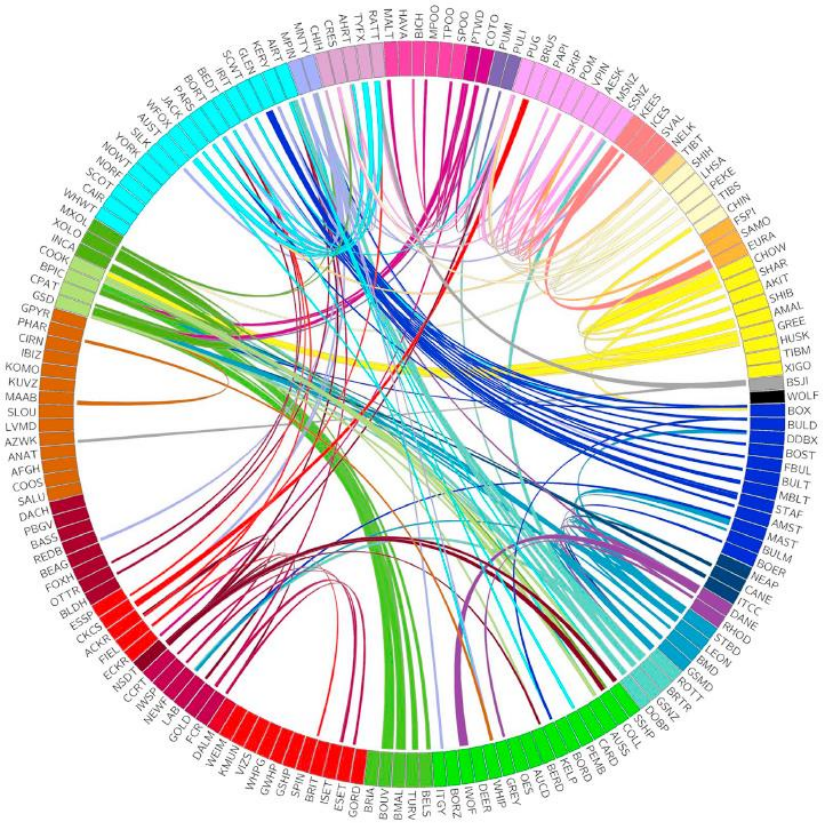

Figure 4 Haplotype sharing between breeds from different phylogenetic clades

The circus plot is ordered and colored to match the tree in Figure 3. Strips connecting breeds represent a median haplotype sharing between all dogs of each breed more than 95% of all haplotype sharing throughout clades. Adapted from Parker et al., 2017 with permission from Elaine A. Ostrander.

6.1. Genetic polymorphism

The variations in a transmitted gene can be rare in a population or can exist in a relatively large number of individuals in a certain population. Genetic polymorphism is defined as genetic variations that occur at a frequency of 1% or more in a population. When we can link a disease to a specific mutation, in some cases, we can provide specific treatments and, in the case of veterinary medicine, guidance about breeding decisions. Despite that, many diseases have more than one gene involved, these are polygenic disease (for example hip dysplasia, epilepsy, etc.). Polygenic diseases are more complex in physiopathology and specific treatments are much difficult to meet (Mealey, 2018).

In both humans and animals, the drug response and the adverse reactions are impaired by several factors such as age, environment, gender and genetic composition of the individual which may be influenced by ancestry or breed, depending on if we are talking about humans or animals. Within the same species, the genetic variation is a consequence of DNA base pair

switch (SNP's), insert or deletion (indels) and if they occur in the coding region of genes, may have significant effects on the translated protein (Syvänen 2001).

6.2. MDR1 and dog breeds

The variability in expression of MDR1 can influence the characteristic pharmacokinetic of the P-gp substrates and this variability occurs on the individual, depending on the tissue, between individuals in the same species and between different species (Martinez et al. 2008b).

Regarding dogs, ABCB1 polymorphism consists on a mutation that leads to a 4 base pairs deletion, result in premature spot codons during transcription. Thus, the translation ends before 10% of the protein is synthesized and, despite being correctly inserted on the cellular membrane, the protein is not functional (Martinez et al. 2008b), so the animals that present two mutant alleles will express a null phenotype to P-gp and will be 100 times more sensitive to develop neurotoxicity after ivermectin administration than dogs that express wild-phenotype. Null-phenotype dogs are equally sensitive to other avermectins such as milbemycin, selamectin and moxidectin as well as to other none avermectin related drugs as loperamide and propofol. Heterozygous dogs present an intermediate phenotype concerning the reaction to avermectins which implies dose adjustments (Mealey and Meurs 2008).

Mealey et al. (2008), investigated vincristine-associated hematological toxicity in dogs with lymphoma and concluded that homozygous (mutant/mutant) and heterozygous dogs were more predisposed to develop hematologic abnormalities than the wild-type homozygous dogs. These authors also report that two of the homozygous mutant dogs died because of complications of sepsis after doxorubicin administration. These two dogs developed severe neutropenia and severe GI toxicity during the 10 days after the treatment. There are other reports of severe vinblastine, vincristine and doxorubicin secondary reactions in Collies and other herding breeds. Therefore, herding breed and mixed dogs should be genotyped before treatment and the dosages should be adjusted based on the MDR1 genotype of the patient.

Typically, this mutation will affect shepherd breeds (Figure 5) and the studies indicate that 75% of the Collies in the United States of America, France and Australia have at least one mutant allele (Mealey and Meurs 2008), however, many other breeds are affected. Such as Longhair Wippet (65%), Australian Shepperd (50%), Miniature Australian Shepperd (50%), McNab Shepperd (30%), Silken Windhound (30%), English Shepperd (15%), Shetland Shepperd (15%), German Shepperd (10%), Old English Sheepdog (5%), Border Collie (<5%) (Mealey and Meurs 2008; Gramer et al. 2011). The animals resulting from crossbreeding of pastoral breeds present a frequency of approximately 10% and crossbred animals 5% (Mealey 2016).

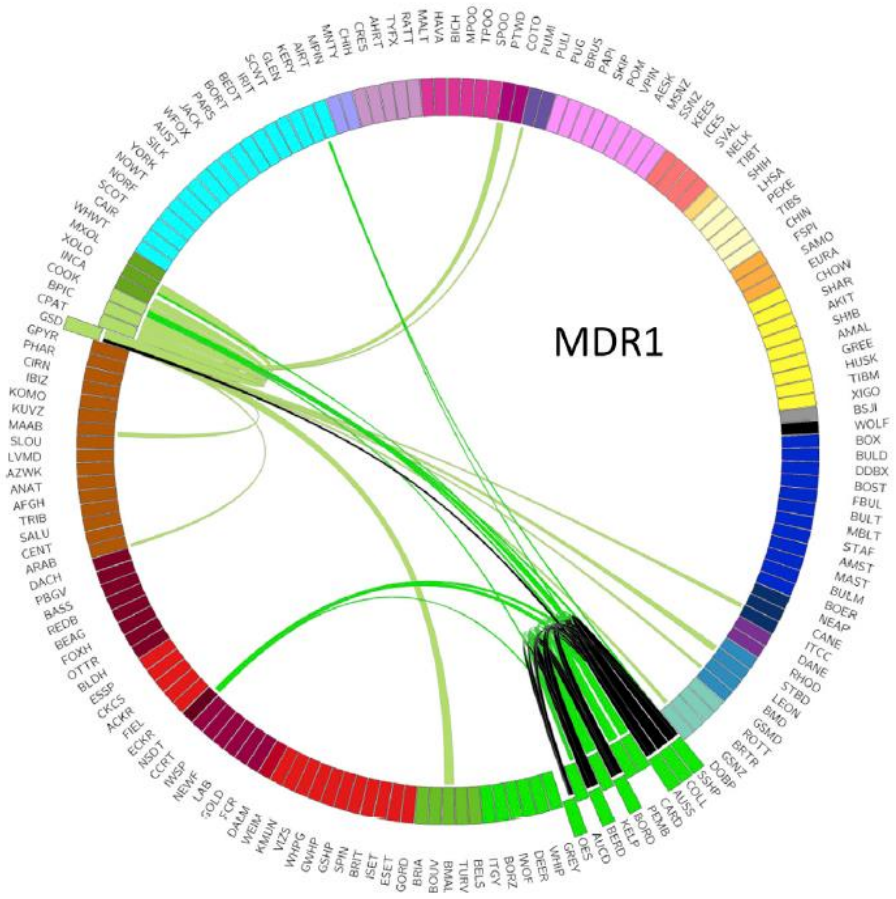

Figure 5 Haplotypes shared with breeds that carry MDR1 mutation

The connected breed has a median shared haplotype size greater than 95% threshold for interclade sharing. Black strips identify the sharing between breeds that are known to carry the mutation sharing between other breeds and are colored equally to the breed that carries the mutation. Adapted from Parker et al., 2017.

Until 2001, it was known that the concentration of ivermectin in brain tissue from ivermectin-sensitive collies was significantly greater than in non-sensitive breeds. Usually, the concentration of ivermectin in brain tissue is 10 and 100 times lower than in plasma and liver concentrations, respectively. In sensitive individuals, the concentration of ivermectin in brain tissue is higher than both plasma and liver concentrations (Pullian *et al*., 1985). Mealey et al. (2001) hypothesized that Collies could be affected by a polymorphism affecting P-gp activity, whether by altering the protein itself or by differences in the levels of its expression. They identified the gene performing semi-quantitative RT-PCR analysis on RNA isolated from sensitive and non-sensitive Collies to determine in which one the expression of MDR1 was lower. The values did not vary significantly between sensitive and non-sensitive collies. Using a RT-PCR, they cloned MDR1 cDNA from one normal dog and three ivermectin-sensitive dogs. Sequence data from the normal dog MDR1 cDNA were equal from those reported for normal canine cDNA. In contrast, sequence analysis of cDNA from the three sensitive Collies shown

a deletion of 4-bp over the first 10% of the transcript (Figure 6). This mutation results in a frame-shift mutation at codon 294 of the transcription, which corresponds to amino acid 75. This mutation leads to several stop codons, the first of it at amino acid position 91, resulting in a truncated, non-functional protein. The remnant sequence of MDR1 cDNA is the same as that of non-sensitive animals. The truncated protein does not have any of the essential components to it work ATP binding sites, substrate binding sites, phosphorylation sites and multiple membrane-spanning motifs (Yoshimura et al. 1989), so it is logical to assume that a functional form of P-gp can never occur in homozygous for the deletion.

Wild-type dog	275 TGGTTTTTGGAAACATGACAGATAGCTTTGCAAATGCAGGAATTTCAAGAAACAAAACTTTT 336
Mutant dog	275 TGGTTTTTGGAAACATGACAG - - - - CTTTGCAAATGCAGGAATTTCAAGAAACAAAACTTTT 336

Figure 6 Comparison of the sequence of the wild-type and mutant dogs

The homozygous dogs for the deletion [MDR1 (mutant/mutant)] demonstrated neurologic effects after a single ivermectin administration, at a dosage of 100µg/kg. Heterozygous (MDR1 wildtype/mutant) while homozygous wildtype dogs do not experience neurologic effects at a 100µg/kg, but they may exhibit signs at higher doses (Mealey 2004).

After this discovery, the allele was found in three Australian Shepherds, one of them exhibited signs of neurotoxicity, which suggests that these two breeds share a common ancestry and that the MDR1 might arise from a single ancestral mutation that has been equally inherited by descent. Other breeds that share the working Collie lineage, such as Shetland Sheepdogs and Old English Sheepdog, had shown ivermectin sensitivity as well as several of herding breeds (Neff et al. 2004).

To determine if there were more breeds at risk of multidrug sensitivity Neff et al. (2004) selectively evaluated dog populations based on phylogeny and phenotype. To establish general baseline of MDR1 frequency trough different pure breeds, they tested four classes of dogs: breeds from the Collie lineage that were selected based on a composite of breed histories; European herding breeds that were not thought to be closely related to the Collie; sighthounds and miscellaneous breeds that had exhibited drug sensitivities, often in response to ivermectin; and a multibreed panel composed for more than 90 breeds. They found that seven breeds from the Collie lineage and two breeds from the sighthound class segregate the mutant allele (Figure 6).

These authors established a distribution among breeds of the gene. They discovered the allele in nine breeds: Australian Shepherd, Collie, already reported, and seven more Australian Shepherd Miniature, English Shepherd, Longhaired Whippet, McNab, Old English Sheepdog, Shetland Sheepdog and Silken Windhound.

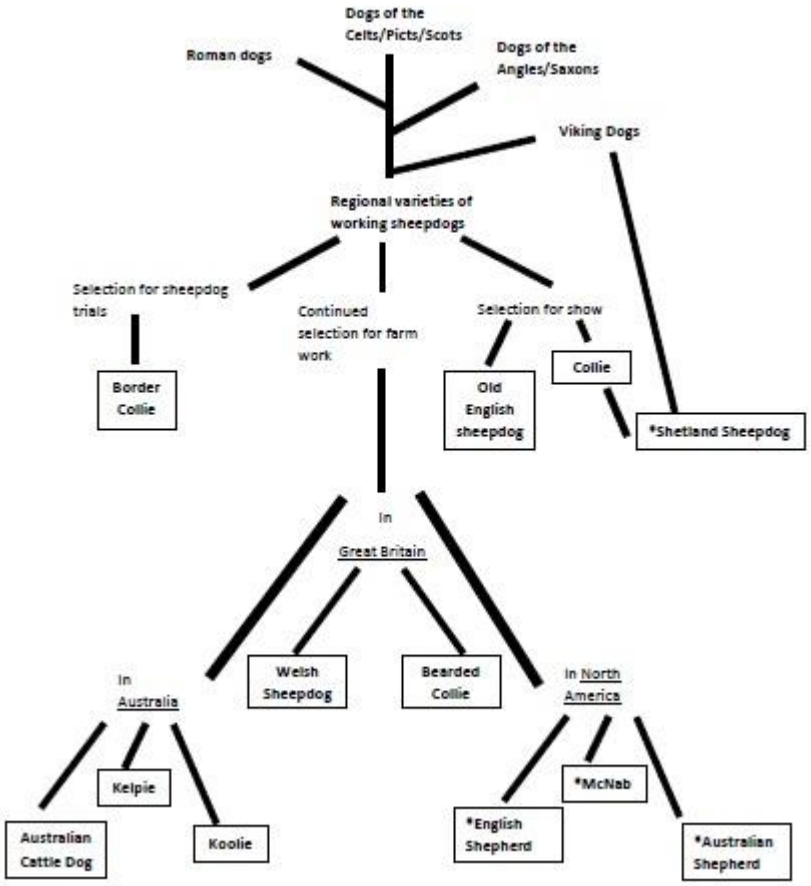

Figure 7 Collie Family Tree. Adapted from Neff et al., 2004

The diagram depicts reported historical relationships among contemporary herding breeds that share the collie lineage out of Great Britain. The breeds shown were selectively surveyed for the presence of MDR1. Breeds that segregated the mutation are shown with an asterisk.

7. Drugs related to neurotoxicity due to MDR1 mutation

When the clinician is treating an animal, from a breed that is known to carry the mutation of gene MDR1, careful should be taken in relation to the drugs listed in Table 1 (Mealey 2016) – these drugs should be avoided or, used at reduced doses. However, the three drugs that are more significant are ivermectin, emodepside and loperamide (Bento 2012). This author, analyzed the data collected from the French veterinary pharmacovigilance system, at Centre de Pharmacovigilance Vétérinaire de Lyon (CPVL). They received a total of 5529 reports between 2005 and 2011, including 170 (3.1%) resulting from ivermectin administration, 154 (2.8%) resulting from emodepside administration and 48 (0.9%) from loperamide administration. However, only 26% of the total canine population with ADRs reported after ivermectin administration belong to sensitive breeds. On the other hand, 73% of the ADRs

reported after loperamide use and 69% of the ADRs reported after emodepside administration belong to breeds that are known to carry the MDR1 gene mutation. The Collie family represents 47% of the ADRs to loperamide and, the Australian Shepperd dogs are involved in 40% of the ADRs to emodepside, reported to CPVL (Bento 2012).

7.1. Macrocyclic Lactones

The Macrocyclic Lactones (MLs), include two groups: avermectins (ivermectin, eprinomectin, selamectin) and milbemycins (milbemycin, moxidectin). They are a complex of chemical-related compounds which have a potent anthelmintic activity (Burg et al. 1979). Milbemycins were first reported by Mishima et al. (1975), for their acaricidal activity, however, the anthelmintic activity was not discovered at the time. The major structural difference between avermectins and milbemycins is the replacement of the macrolide ring of the avermectins with a disaccharide group at the 13 position in milbemycins. Others variations in the structures of the natural substances are confined to substituents at carbon atoms (Fisher and Mrozik 1984).

It is well known that the major differences between vertebrates and insects lay in the nervous system. One of the most important differences is related to the cholinergic nature of the motoneurons regulating vertebrate skeletal muscle, compared with the gamma-aminobutyric acid (GABAergic) and/or glutaminergic nature of motoneurons regulating skeletal muscle activity in insects and other invertebrates. Also, invertebrates have glutamate-gated chloride channels that do not exist in mammalian systems. The fact that GABAergic and glutamate receptors are only found in the CNS of the mammalians systems, allows us to discover and use compounds that reach these receptors in insect muscles, but not penetrate in the BBB (Jansson and Dybas 1998).

Avermectins are extremely lipo-soluble and bind tightly to various tissues and proteins, so it is not possible to know for sure the actual concentration of the compounds at the active site of isolated organs. When administered to animals, avermectins will enter the bloodstream and bind to plasma proteins and be distributed throughout the body, achieving high concentrations in the liver but comparatively low concentrations in the brain, because of BBB and apparently it is this compartmentalization that differentiates toxicity against invertebrates and mammalian (Fisher and Mrozik 1984).

7.2. Emodepside

Emodepside is a cyclic octadepsipeptide, a new class of antiparasitic drugs, that is a semisynthetic derivate of PF1022A, a secondary metabolite of fungus *Mycellia sterilia* (Harder et al. 2005; Elmshäuser et al. 2015). This new drug is used to treat gastrointestinal nematodes infestation in dogs and cats (EMEA, 2008).

During the approval of this drug, it was identified as a substrate of P-gp (EMEA, 2005). Knowing the relevance of P-gp on the therapeutic with antiparasitic drugs, Elmshäuser et al. (2015) investigated if it was also important to emodepside, administering this compound orally to MDR1 deficient (PGP^{mut}) and MDR1 intact (PGP^{WT}) mice at 1mg/kg and analyzing the brain penetration and appearance of neurotoxicity. When they analyzed the brain penetration of emodepside in the PGP^{WT} mice, the concentration of the drug was below the methodological detection level (10ng/g), but in the PGP^{mut} mice, the mean concentration was at 43.7ng/g (emodepside as detected in the brain of all mutant mice). This finding allows the authors to affirm that the P-gp has a functional rule for limiting the penetration of emodepside into the brain and indicate that the bioavailability of emodepside was not significantly affected in the PGP^{mut} mice (Elmshäuser et al. 2015).

7.3. Loperamide

Loperamide is a piperidine derivate, chemically related to diphenoxylate and pethidine (Mellstrand 1987). It is a highly dependent P-gp substrate that is commonly used to treat diarrhea in dogs, considering that its effects are normally restricted to the gut, with no effects on CNS despite its potent opioid effect *in vitro*. This difference between *in vitro* and *in vivo* action and apparent tissue selectivity is probably due to the efflux of loperamide by P-gp which prevents access of loperamide to the CNS. This hypothesis is supported by the fact that in P-gp knockout mice, brain loperamide concentrations were eight times higher than in wild-type mice, producing lethal opioid effects (Wandel et al. 2002). However, P-gp expression does not affect the transcellular movement of other opioids such as fentanyl, sufentanil or alfentanil, which shows a relative dependence of it. These differences are due to the high-affinity P-gp substrate that is so efficiently pumped out of the CNS for it that the concentrations achieved in the brain do not have pharmacological relevance (Wandel et al. 2002).

Zhu et al. (2016), evaluated, at the genomic level, the impact of the ABCB1 mutation associated with ivermectin sensibility, on drug safety of loperamide in Collie dogs that were homozygous for wild-type, heterozygous and homozygous mutant. They examined the alterations on gene expression induced by loperamide in dogs with known phenotypes of

ivermectin sensitivity, using whole genome gene expression microarray. Furthermore, they analyzed the altered genes for association with gene pathways and structure and identified potential biomarkers to predict sensitivity and toxicity of loperamide in ABCB1 mutant Collies. When they administered escalating doses of loperamide to analyze the phenotype response (CNS toxicity) the changes start to appear at the dosage of 0.10mg/kg loperamide. At this dosage, three of the non-sensitive Collies showed salivation or salivation and mydriasis, the ivermectin-sensitive Collies exhibited salivation, mydriasis, ataxia and depression and they found that loperamide treatment causes the main difference in gene expression, mostly in the sensitive Collies.

As a conclusion in the absence of functional P-gp, the alterations produced in the BBB potentially cause elevated drug levels in the brain, decreased drug elimination and increased drug toxicity (Schinkel et al. 1994) and it affects loperamide pharmacokinetics producing neurologic signs.

7.4. Symptoms of neurotoxicity in dogs

Ivermectin use is indicated for generalized demodicosis, sarcoptic acariasis or cheyletiellosis in dogs and the doses range from 200µg to 600µg/kg, *per os* (Mealey, 2004; Ramsey & British Small Animal Veterinary Association, 2017). Despite that, in Portugal, the use is only indicated for *Dirofilaria immitis, Toxocara canis, Toxascaris leonine, Ancylostoma caninum* and *Uncinaria stenocephala*. However, the guidelines advert that the use of ivermectin is not recommended in Collies and related breeds and explicit that neurotoxicity may be seen if the drug crosses the BBB.

Commonly, signs of intoxication with macrocyclic lactones are associated with the CNS and include neurologic depression, hypersalivation, tremors, mydriasis, ataxia and blindness. Seizures may also occur. The signs can persist for days or even weeks, depending on the dose and the breed involved (Merola and Eubig 2012).

A specified pharmacotherapy might be considered for other drugs and P-gp substrates such as vincristine and doxorubicin. On the other hand, using decreased doses may lead to shorter remission times as remission periods tend to be depending on drug dosage (Mealey 2004).

III. The MDR1 mutation in canine breeds in Portugal – experimental study

1. Objectives

The presence of the MDR1 mutation can have serious implications on the clinical daily practice so, is important to know its prevalence in the canine population. As far as we know, there are no studies made in Portugal about the prevalence of this mutation either in the breeds known as affected by the mutation and in the indigenous breeds.

According to the *Federation Cynologique Internationale* (FCI), breeds that are recognized on a definitive basis are organized in 10 groups based on the breed characteristics. Most of the breeds that are affected by MDR1 mutation belong in group 1 as well as Cão da Serra de Aires (Attachment 1). Although, Barbado da Terceira is not a recognized breed to FCI is classified by Clube Português de Canicultura as a group 1 breed (Carlos and Magalhães, no date). Thus, we thought that these two breeds should be tested for MDR1 mutation. For test validation, we also tested dogs that had already been tested and some dogs from predisposed breeds that needed to be tested.

Therefore, the objectives of this work were to implement the laboratory technique to detect the mutation, to test the presence of the mutation on a sample of the population of Barbado da Terceira and Cão da Serra de Aires and on a sample of the population of herding breeds in Portugal known as being affected by the mutation.

The study was submitted and approved by the Comissão de Ética e Bem Estar Animal (CEBEA) of Faculdade de Medicina Veterinária.

2. Material & Methods

2.1. Animals

For sampling purpose, clinically healthy animals were chosen. Samples were collected from Lisboa e Vale do Tejo region and Sintra region from known breeders, in dog shows and without restriction. We have some samples from dogs that do not belong to Group I, just to increase our sample.

2.2. Biological Samples

The samples were collected randomly by venipuncture (8) and oral swabs (97) in the owner's house or in the dog shows and stored frozen until the DNA extraction.

2.3. DNA extraction and PCR

DNA from blood samples and oral swabs was extracted using the High Pure PCR Template Preparation Kit (Roche®) following the kit instructions.

For the 50µl PCR, 20µl of extracted DNA was used as a template for polymerase chain reaction (PCR) amplification under the conditions at 94ºC for 4 minutes; 32 cycles consisting of 94ºC x 30 seconds, 55ºC x 1 minute, 72ºC x 30 seconds; followed by 72ºC for 10 minutes. Each PCR reaction tube contained reaction buffer 5x, $MgCl_2$, dNTP, both primers, 2.5% DMSO, Taq polymerase and H_2O. The primer sequences were as follows: forward primer 5' – GGC TTG ATA GGT TGT ATA TGT TGG TG – 3'; reverse primer 5' – ATT ATA ACT GGA AAA GTT TTG TTT – 3'.

After the PCR, we ran a 2% agarose gel to be sure that the quantity of DNA was enough, so we can proceed with the purification to sequencing.

2.4. Purification

To purify the PCR product samples, the kit DNA Clean & Concentrator™-5 from Zymo Research was used following the kit instructions. In a microcentrifuge the PCR product sample was mixed with the binding buffer and mixed briefly by vortexing. Then, the mixture was transfere to the column in a collection tube, centrifugated and discard the flow-through. After this procedure, the wash buffer was added to the column and centrifugated. Thereafter, the column was transfer to a microcentrifuge tube, added the wash elution buffer, incubate at room temperature and centrifugated to elute the DNA.

2.5. DNA sequencing

The DNA sequencing was made by sanger sequence in STAB VIDA, an external laboratory belonging to Faculdade de Ciência e Tecnologias (FCT) da Universidade Nova de Lisboa.

3. Results

The composition of the sample is described in Table 3. We had a total of 7 blood samples and 98 oral swabs.

Table *3* Sample summary by breed

Breed	Total
Australian Shepherd	32
Barbado da Terceira	23
Bearded Collie	4
Belgian Shepherd	1
Border Collie	7
Cão da Serra de Aires	10
German Shepherd	3
Jack Russel	6
Labrador Retriever	7
Rough Collie	4
Swiss Shepherd x Border Collie	8
Total	**105**

As can be seen in Table 4, a total of 105 dogs from Portugal were MDR1 genotyped - eight purebred dogs breeds and one crossbred. More than a third of all samples were derived from Australian Shepherds and Border Collies. Samples from Barbado da Terceira and Cão da Serra de Aires (our target breeds) accounted approximately 31% of the tested specimens.

The mutant allele was detected in three purebred dog breeds – Australian Shepherd, Barbado da Terceira and Rough Collie - and in the crossbreed dogs. As can be seen in the same table, the MDR1(-) allele was identified in Barbado da Terceira individuals, although we did not identify any homozygous MDR1(-/-) individual.

Table 4 Frequencies of MDR1 mutation in Portuguese dogs

Breed	Heterozygous (-/+)	Mutant (-/-)	Wild type (+/+)	Total
Australian Shepherd	19 (59%)	4 (12.5%)	9 (28.1%)	32
Barbado da Terceira	7 (30.4%)		16 (69.6%)	23
Bearded Collie			4 (100%)	4
Belgian Shepherd			1 (100%)	1
Border Collie			7 (100%)	7
Cão da Serra de Aires			10 (100%)	10
German Shepherd			3 (100%)	3
Jack Russel Terrier			6 (100%)	6
Labrador Retriever			7 (100%)	7
Rough Collie		3 (75%)	1 (25%)	4
Swiss Shepherd x Border Collie	2 (25%)		6 (75%)	8
Total	**28**	**7**	**70**	**105**

The frequency of the mutant allele differs between these dog breeds: 71,8% of the Australian Shepherds and 75% of the Rough Collies analyzed showed at least one mutant

MDR1(-) allele, which occurred either in the homozygous MDR1 (-/-) or the heterozygous MDR1 (-/+). In Barbado da Terceira and mixed dogs, the total account is 30.4% and 25%, respectively. We did not find the mutant allele in the other tested genotyped breeds (Bearded Collie, Belgian Shepherd, Border Collie, Cão da Serra de Aires, German Shepherd, Jack Russel and Labrador Retriever). However, we know that we only tested a few individuals of each breed and that we have a restrict sample.

Table 5 resumes the MDR1 genotyping data from herding breeds worldwide.

Table 5 Frequencies of the MDR1 mutation in different countries

		US[a]	US[b]	Germany[c]	UK[d]	France[e]	Japan[f]	Australia[g]	Israel[g]	This study
Australian Shepherd	MDR1 +/+	53.0%	68.5%	67.9%	32.1%		44.4%	35.7%	46.2%	28.1%
	MDR1 -/+	37.0%	29.8%	25.2%	42.9%		44.4%	42.8%	44.8%	59%
	MDR1 -/-	10.0%	1.7%	6.9%	25.0%		11.2%	21.5%	9.0%	12.5%
Border Collie	MDR1 +/+	98.4%	100%	99.1%	95.3%				92.7%	100%
	MDR1 -/+	1.3%	0%	0.6%	4.7%				5.0%	0%
	MDR1 -/-	0.3%	0%	0.3%	0%				2.3%	0%
Rough Collie	MDR1 +/+								50.0%	25%
	MDR1 -/+								45.0%	0%
	MDR1 -/-								5.0%	75%
Collie	MDR1 +/+	22.6%	26.0%	23.9%	7.1%	20.0%	25.0%	12.1%		0%
	MDR1 -/+	42.0%	46.0%	43.1%	40.5%	32.0%	33.3%	63.6%		0%
	MDR1 -/-	35.4%	28.0%	33.0%	52.4	48.0%	41.7%	24.3%		0%

(a) (Mealey and Meurs 2008)
(b) (Neff et al. 2004)
(c) (Geyer et al. 2005)
(d) (Tappin et al. 2012)
(e) (Hugnet et al. 2004)
(f) (Kawabata et al. 2005)
(g) (Mealey et al. 2005)
(h) (Dekel et al. 2017)

On the figures 8, 9 and 10 we can see examples of the wild type, heterozygous and homozygous mutant sequences, respectively.

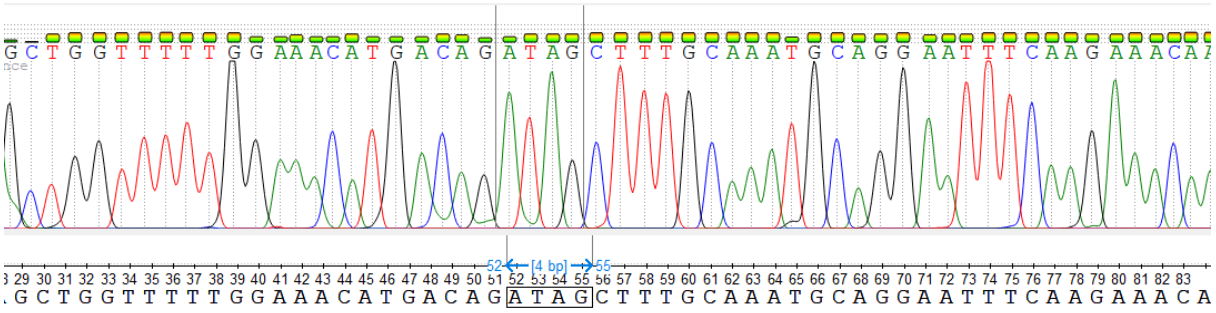

Figure 8 Wild type sequence.

Neither of the two alleles contains the mutation, consequently their sequence is identical. Thus, the peak of each nucleotide is tall and distinct.

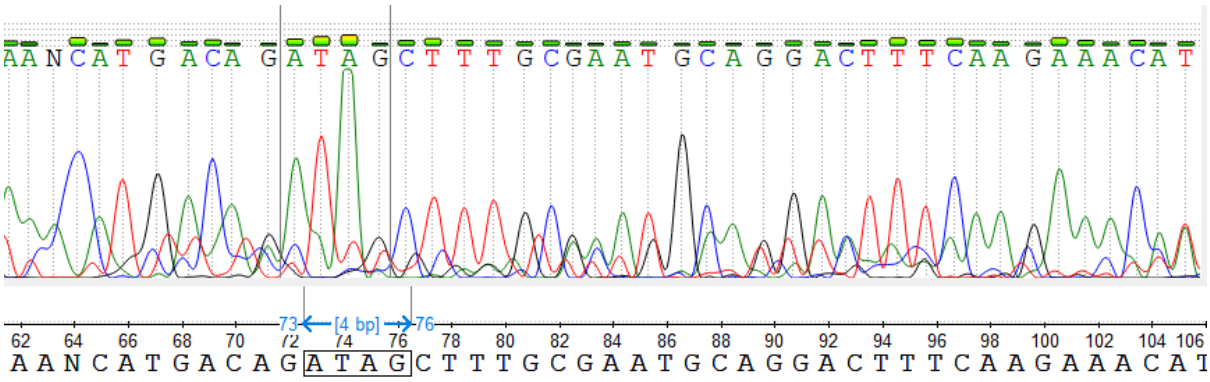

Figure 9 Heterozygous sequence.

One complete allele and one allele carrying the deletion mutation, causing distortion of the sequence output from that point (two peacks). In this way, we see a C peak from de mutant sequence under the A peak from the complete allele, a bigger T peak because of the overlap between the two alleles, and a second A peak much bigger than in the wildtype sequence, also because of the overlapping.

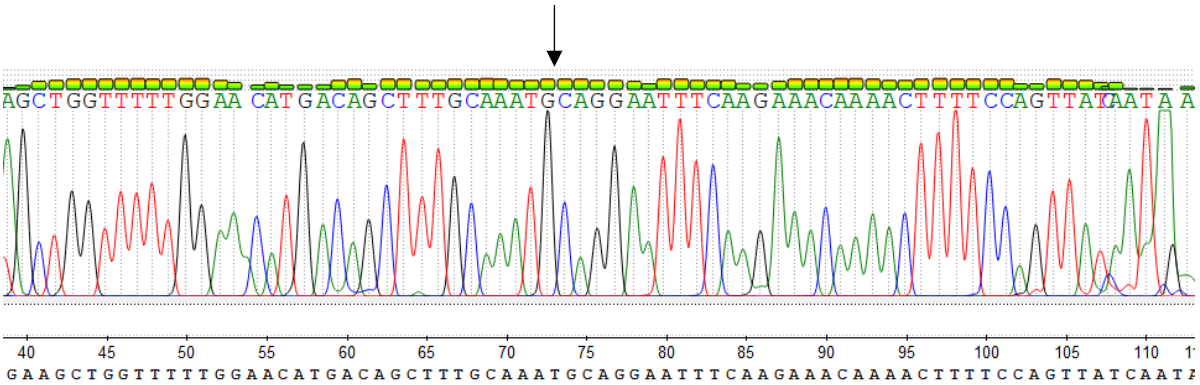

Figure 10 Homozygous mutant sequence.

Both alleles have the mutation, thus they are identical. The arrow indicates the position where the sequence is missing.

4. Discussion and conclusions

Before a company can place a VMP in the market, a Marketing Authorization (MA) is needed, under European Union (EU) or national legislation. The MA can only be approved after laboratory, preclinical and clinical trials. However, these trials are limited in time, cost and rationalization, leading to tests performed directly on target species and in a small number of animals. Once in the market, the VMP will be applied to an undefined number of animals under different clinical conditions. Even within the same species, it will be applied to several breeds with their metabolic and sensitive characteristics, with different pathologies, in different ages, with medical history and, possibly, other drug therapy. Moreover, the drug, in most cases, will not be administered only by professionals, but also by the owners. Consequently, only the daily practice conditions can establish the drug safety and the pharmacovigilance should allow the

collection of each suspected adverse reaction in animals, on the people in contact with the animal and in the environment.

When a new veterinary medicinal product (VMP) is released into the market, adverse drug reactions that were unknown, may become apparent. These reactions can be seen in the animal, in exposed users or in the environment. Additionally, these reactions might be obvious from excess of drug residues in animal products such as milk and meat. Thus, it is import to establish legislation and regulatory approaches across the world to respond to these issues, ensure and monitoring the safety of these products and, when necessary, take regulatory measures (Woodward 2009).

The World Health Organization (WHO) defines pharmacovigilance as the science and activities related to detection, evaluation, comprehension and prevention of the adverse effects, or other problems related to drug administration being its main function to guarantee that the treatments are safe (Härmark and Van Grootheest 2008).

In Europe, the regulatory authority is the European Medicines Agency (EMA), which centralizes the information, issues guidelines about drug use and publish annually, public bulletins regarding the results of pharmacovigilance. According to EMA, this latter also includes reports about side effects related to off-label usage, investigation that validates market withdrawal period and potential environmental problems (2019).

It is the responsibility of the clinicians to report adverse reactions and the pharmacovigilance authorities should collect, perform, evaluate and record the cases. The analysis of these cases can offer a better knowledge of possible side effects, their frequency, risk factors, occurrence and prognosis concerning to a particular specie or breed (Woodward 2009). For example, the fact that there was a subpopulation of Collie dogs extremely sensitive to neurotoxicity induced by ivermectin lead to the discovery of the MDR1 mutation, and the report of the same adverse reaction in individuals from another herding breeds extended the research to them worldwide. This was only possible because of the reports and data analyses by the EMA.

The clinicians should be aware that no antidote is available, and this intoxication may lead to death. They should “treat the patient and not the toxicant”. The great majority of the patients require close monitoring, a symptomatic intervention and a good nursing care. There are 3 basic principles in intoxication treatment: avoid further absorption, supportive therapy and specific antidotes (when they are available) (Bento 2012).

In this specific case, the absorption can be stopped by provoking emesis within a few hours of ingestion, however, is contraindicated in dogs without swallowing reflex, when the animal is convulsing or when there is an imminent risk of aspiration pneumonia. Oral emetics include hydrogen peroxide (2ml/kg, *per os*), syrup of ipecac (10-20ml, *per os* in dogs) and apomorphine (0.05-0.1mg/kg), via parenteral. A gastric lavage can be performed on

anesthetized or unconscious animals. When the toxicant cannot be physically removed, several substances can be administered orally to adsorb it and prevent its absorption. The most usually used is activated charcoal (1-2g/kg) (Bento 2012).

The supportive care differs with the patient status, but normally includes anticonvulsants, maintenance of the ventilation, fluid therapy, electrolyte reposition, treatment of shock, control of cardiac dysfunction and pain management.

Besides the ivermectin's reactions, more recently, were also identified the same type of reactions after the administration of emodepside (Gaens et al. 2019). In fact, in our hospital, we had an Australian Shepherd dog presented with the same neurological signs (generalized tremor, agitation and panting) in 2018 and, the MDR1 genotyping revealed a homozygous mutation of the MDR1 gene. These cases highlight the importance of MDR1 genotyping in predisposed dog breeds and the importance of the clinics to be aware for this mutation. Furthermore, is also important that the veterinarians know how to treat these dogs with P-gp substrates and what dosages can be used, as described in Table 6.

According to the CPC (Clube Português de Canicultura) official data from 2017, there were a total of 36 Australian Shepherd (88.8% tested in this study), 110 Barbado da Terceira (20.1% tested in this study), 316 Belgian Shepherd (0.32% tested in this study), 61 Border Collie (11.4% tested in this study), 81 Cão da Serra de Aires (12.3% tested in this study), 1754 German Shepherd (0.17% in this study), 217 Jack Russel Terrier (2.76% tested in this study), 1274 Labrador Retriever (0.55% tested in this study) and 44 Rough Collie (9% tested in this study) registered. Because of the new data privacy policy, we cannot have access to the number of registered dogs in 2019, wherefore, more dogs can be registered and not included in our statistic. Thus, one of the limitations of the present study is that the sampling is not representative of the all population in all the breeds. Besides that, we did demonstrate that the mutant allele is present in the Barbado da Terceira population. In this regard, we cannot say that the mutation does not exist on the Cão da Serra de Aires population. We only can say that the mutation is present on the Barbado da Terceira's population.

Table 6 How to treat animals with MDR1 mutation (Mealey)

Drug	Effect in dogs with MDR1 mutation	How to treat:	
		MDR1 (-/-)	MDR1 (+/-)
Acepromazine	Profound and prolonged sedation	30% to 50% reduction	25% reduction
Butorphanol	Profound and prolonged sedation	30% to 50% reduction	25% reduction
Doxycycline	Is safe at normal dosage		
Erythromycin	Neurologic toxicity	Should be avoided	No information available
Emodepside + Praziquantel	Neurologic toxicity	Should be avoided	No information available
Ivermectin	Neurologic toxicity	Should be avoided	
Milbemycin, Moxidectin, Selamectin	Neurologic toxicity	Should be avoided	No information available
Doxorubicin, Actinomycin D	Risk of neutropenia, thrombocytopenia and GI adverse effects	Initial dose reduction of 50%, with subsequent doses increased as tolerated	Initial dose reduction of 25%, with subsequent doses increased as tolerated
Paclitaxel, Docetaxel	Severe myelosuppression in MDR1 (+/-) dogs	Should be avoided	
Vinblastine, vincristine, Vinorelbine (Vinca Alkaloids)	Increased risk of neutropenia, thrombocytopenia, GI and neurologic effects, neurologic toxicity	50% reduction	25% reduction
Loperamide	Neurologic toxicity	Should be avoided	No information available
Ondansetron	Mild-to-severe CNS depression	Should be avoided	

Comparing genotype frequencies, described in Table 5, regarding Australian Shepherd dogs, we obtained a frequency of the wildtype genotype similar to the English (Tappin et al. 2012) and Australian (Mealey et al. 2005) studies. The frequency of homozygous mutant individuals is very similar to the Japanese (Kawabata et al. 2005) and American (Mealey and Meurs 2008) studies. However, the frequency of the heterozygous individuals is higher than any other. Probably, this discrepancy on the heterozygous dog's frequency results from the breeding of individuals that are heterozygous or with an unknown genotype.

Although we have a non-representative sampling of Border Collie, we do have a frequency of wildtype (100%) individuals that is equal to Neff et al. (2004), in the United States, and is very close to the frequency obtained in Germany by Geyer et al. (2005). These results

probably emphasized the care of the breeders, to not reproduce heterozygous or mutant individuals.

Regarding the breeds that do not belong to Group I, we did not expect to identify the mutation as it happened.

The existence of this mutation in Barbado da Terceira and Cão da Serra de Aires had never been done before. We did it because of the importance of this mutation in the course of treatment and, since these two breeds belong to group I in the FCI classification, we thought that they might carry the mutation. In fact, we identified 6 (28.6%) Barbado da Terceira that are affected by the mutation, all of them are heterozygous.

One of the dogs that are heterozygous, had an anaphylactic reaction after being treated with Advantix® and Drontal® simultaneously. The dog became prostrate and vomited. Never had a convulsive episode. The clinician administered dexamethasone and told the owner to give him a bath. A few hours later the dog was perfectly normal. This can be just a coincidence, since none of the administered drugs has substance known to be a P-gp substrate. However, we still know little about the reactions in heterozygous dogs and there might be other substances that, in fact, are substrates for P-gp and we do not know. Vomit is centrally mediated so, maybe there is a link between the reaction and the fact that the dog is heterozygous for the MDR1 mutation.

It is very important to inform the breeders about this mutation and highlight the fact that they should test the dogs before breeding, even when they inform the owners about the risks and the drugs that should not be given to these animals. The only way to prevent the mutation to perpetuate is not breeding these dogs.

It is well established that the ample amount of the dogs that are sensitive to P-gp substrates are affected by a single mutation. On the other hand, in cats there may be a combination of factors or a mutation that was not identified that may explain the neurologic signs after the ivermectin administration. It would be expected that the cats that are affected by the mutation have an identical phenotype as dogs with MDR1 mutation, presenting enhanced susceptibility to adverse effects of P-gp substrate drugs such as vinca alkaloids, macrocyclic lactones, doxorubicin, loperamide among others. And, like dogs, it would be expected that heterozygous cats have an intermediate phenotype. However, more research is needed to know the association of this deletion with adverse reactions to P-gp substrates in cats (Mealey and Burke 2015).

To conclude, it is important to emphasize, once more, that the clinicians should be aware of this mutation and its implications on drug therapy, so they can avoid adverse reactions. Additionally, is important to report any reaction that can be associated with drug administration to the national entities, so they can report it to EMA. This organization will collect the information and send out bulletins and studies about the causes of the reaction and with

any important measure to reduce adverse drug reactions, leading, eventually, to market withdrawal of the concerning drug.

IV. Bibliography

Abbott NJ. 2005. Dynamics of CNS Barriers: Evolution, Differentiation, and Modulation. Cell Mol Neurobiol. 25(1):5–23. doi:10.1007/s10571-004-1374-y.

Abbott NJ, Patabendige AAK, Dolman DEM, Yusof SR, Begley DJ. 2010. Structure and function of the blood–brain barrier. Neurobiol Dis. 37(1):13–25. doi:10.1016/j.nbd.2009.07.030.

Bento R. 2012. VETERINARY PHARMACOVIGILANCE, FROM REGULATION TO SCIENTIFIC EXPLANATION. CASE STUDIES OF CANINE MDR1 MUTATION. - Dissertação de Mestrado, Faculdade de Medicina Veterinária - Universidade de Lisboa.

Boron WF, Boulpaep EL. 2012. Organization of the nervous system. In: Medical Physiology, 2e Updated Edition E-Book: with STUDENT CONSULT Online Access. 2nd ed. Elsevier Health Sciences.

Burg RW, Miller BM, Baker EE, Birnbaum J, Currie SA, Hartman R, Kong Y-L, Monaghan RL, Olson G, Putter I, et al. 1979. Avermectins, New Family of Potent Anthelmintic Agents: Producing Organism and Fermentation. Antimicrob Agents Chemother. 15(3):361–367. doi:10.1128/AAC.15.3.361.

Carlos RF, Magalhães RDA. Clube Português de Canicultura. http://www.cpc.pt/cpc/ag/docs/20171122_Est_Barbado_Terceira.pdf

Coelho JC, Tucker R, Mattoon J, Roberts G, Waiting DK, Mealey KL. 2009. Biliary excretion of technetium-99m-sestamibi in wild-type dogs and in dogs with intrinsic (ABCB1-1Δ mutation) and extrinsic (ketoconazole treated) P-glycoprotein deficiency. J Vet Pharmacol Ther. 32(5):417–421. doi:10.1111/j.1365-2885.2009.01068.x.

Court MH. 2007. A pharmacogenomics primer. J Clin Pharmacol. 47(9):1087–1103. doi:10.1177/0091270007303768.

Court MH. 2013a. Canine Cytochrome P-450 Pharmacogenetics. Vet Clin North Am Small Anim Pract. 43(5):1027–1038. doi:10.1016/j.cvsm.2013.05.001.

Court MH. 2013b. Feline Drug Metabolism and Disposition. Vet Clin North Am Small Anim Pract. 43(5):1039–1054. doi:10.1016/j.cvsm.2013.05.002.

Daneman R, Prat A. 2015. The Blood–Brain Barrier. Cold Spring Harb Perspect Biol. 7(1):a020412. doi:10.1101/cshperspect.a020412.

Dekel Y, Machluf Y, Stoler A, Aderet A, Baumel D, Kellerman E, Plotsky Y, Noked Partouche O, Elhalal G, Ben-Shlomo I, et al. 2017. Frequency of canine nt230(del4) MDR1 mutation in prone pure breeds, their crosses and mongrels in Israel - insights from a worldwide comparative perspective. BMC Vet Res. 13(1):333. doi:10.1186/s12917-017-1251-9.

Dürr D. 2000. St John's Wort induces intestinal P-glycoprotein/MDR1 and intestinal and hepatic CYP3A4. Clin Pharmacol Ther. 68(6):598–604. doi:10.1067/mcp.2000.112240.

Elmshäuser S, Straehle LC, Kranz J, Krebber R, Geyer J. 2015. Brain penetration of emodepside is increased in P-glycoprotein-deficient mice and leads to neurotoxicosis. J Vet Pharmacol Ther. 38(1):74–79. doi:10.1111/jvp.12149.

Ernest S, Bello-reuss EN. 1997. Expression of MDR1 (Multidrug Resistance) Gene and its protein in normal human kidney nephron. 1997;77(3):284-9. DOI: 10.1159/000190289

Fisher MH, Mrozik H. 1984. The Chemistry and Pharmacology of Avermectins. Annu Rev Pharmacol Toxicol. 1992;32:537-53. DOI: 10.1146/annurev.pa.32.040192.002541

Gaens D, Leithäuser C, Hamann M, Geyer J. 2019. Adverse Drug Reactions After Administration of Emodepside/Praziquantel (Profender®) in an MDR1-Mutant Australian Shepherd Dog: Case Report. Front Vet Sci. 6:296. doi:10.3389/fvets.2019.00296.

Geyer J, Doring B, Godoy JR, Leidolf R, Moritz A, Petzinger E. 2005. Frequency of the nt230 (del4) MDR1 mutation in Collies and related dog breeds in Germany. J Vet Pharmacol Ther. 28(6):545–551. doi:10.1111/j.1365-2885.2005.00692.x.

Ginsburg GS, Willard HF. 2016. Genomic and Precision Medicine: Foundations, Translation, and Implementation. Academic Press.

Gottesman MM, Pastant I, Ambudkar SV. 1996. P-glycoprotein and multidrug resistance. Curr Opin Genet Dev. 1996 Oct;6(5):610-7. DOI: 10.1016/s0959-437x(96)80091-8

Gramer I, Leidolf R, Döring B, Klintzsch S, Krämer E-M, Yalcin E, Petzinger E, Geyer J. 2011. Breed distribution of the nt230(del4) MDR1 mutation in dogs. Vet J. 189(1):67–71. doi:10.1016/j.tvjl.2010.06.012.

Greiner B, Eichelbaum M, Fritz P, Kreichgauer H-P, von Richter O, Zundler J, Kroemer HK. 1999. The role of intestinal P-glycoprotein in the interaction of digoxin and rifampin. J Clin Invest. 104(2):147–153. doi:10.1172/JCI6663.

Harder A, Holden–Dye L, Walker R, Wunderlich F. 2005. Mechanisms of action of emodepside. Parasitol Res. 97(S1):S1–S10. doi:10.1007/s00436-005-1438-z.

Härmark L, Van Grootheest AC. 2008. Pharmacovigilance: Methods, recent developments and future perspectives. Eur J Clin Pharmacol. 64(8):743–752. doi:10.1007/s00228-008-0475-9.

Hugnet C, Bentjen SA, Mealey KL. 2004. Frequency of the mutant MDR1 allele associated with multidrug sensitivity in a sample of collies from France. J Vet Pharmacol Ther. 27(4):227–229. doi:10.1111/j.1365-2885.2004.00585.x.

Irion DN. 2003. Analysis of Genetic Variation in 28 Dog Breed Populations With 100 Microsatellite Markers. J Hered. 94(1):81–87. doi:10.1093/jhered/esg004.

Jancova P, Anzenbacher P, Anzenbacherova E. 2010. PHASE II DRUG METABOLIZING ENZYMES. Biomed Pap. 154(2):103–116. doi:10.5507/bp.2010.017.

Jansson RK, Dybas RA. 1998. Avermectins: Bioehemieal Mode of Action, Biologieal Activity and Agrieultural Importanee. In Insecticides with Novel Modes of Action Mechanisms and Application. Editors Isaac Ishaaya and Danny Degheele.

Juliano RL, Ling V. 1976. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta BBA - Biomembr. 455(1):152–162. doi:10.1016/0005-2736(76)90160-7.

Juranka PF, Zastawny RL, Ling V. 1989. P-glycoprotein: multidrug-resistance and a superfamily of membrane-associated transport proteins. FASEB J. 3(14):2583–2592. doi:10.1096/fasebj.3.14.2574119.

Kawabata A, Momoi Y, Inoue-Murayama M, Iwasaki T. 2005. Canine mdr1 Gene Mutation in Japan. J Vet Med Sci. 67(11):1103–1107. doi:10.1292/jvms.67.1103.

Keppler D, Arias IM. 1997. Hepatic canalicular membrane. Introduction: transport across the hepatocyte canalicular membrane. FASEB J. 11(1):15–18. doi:10.1096/fasebj.11.1.9034161.

Kidd LB, Salavaggione OE, Szumlanski CL, Miller JL, Weinshilboum RM, Trepanier L. 2004. Thiopurine Methyltransferase Activity in Red Blood Cells of Dogs. J Vet Intern Med. 2004 Mar-Apr;18(2):214-8. DOI: 10.1892/0891-6640(2004)18<214:tmairb>2.0.co;2

Kim RB, Fromm MF, Wandel C, Leake B, Wood AJ, Roden DM, Wilkinson GR. 1998. The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. J Clin Invest. 101(2):289–294. doi:10.1172/JCI1269.

Klein BG, Cunningham J. 2012. Neurophysiology. In: Cunningham's Textbook of Veterinary Physiology.

Koskinen MT. 2003. Individual assignment using microsatellite DNA reveals unambiguous breed identification in the domestic dog. Anim Genet. 2003 Aug;34(4):297-301. DOI: 10.1046/j.1365-2052.2003.01005.x

Kwei GY, Alvaro RF, Chen Q, Jenkins HJ, Wang RW, Wang Z, Pippert TR, Umbenhauer DR. 1999. DISPOSITION OF IVERMECTIN AND CYCLOSPORIN A IN CF-1 MICE DEFICIENT IN MDR1A P-GLYCOPROTEIN. Drug Metab Dispos. 1999 May;27(5):581-7.

Kwon Y, Kamath AV, Morris ME. 1996. Inhibitors of P-Glycoprotein-Mediated Daunomycin Transport in Rat Liver Canalicular Membrane Vesicles. J Pharm Sci. 85(9):935–939. doi:10.1021/js9600540.

Lindner S, Halwachs S, Wassermann L, Honscha W. 2013. Expression and subcellular localization of efflux transporter ABCG2/BCRP in important tissue barriers of lactating dairy cows, sheep and goats. J Vet Pharmacol Ther. 36(6):562–570. doi:10.1111/jvp.12045.

Maran BA, Mealey KL, Lahmers SM, Nelson OL, Meurs KM. 2013. Identification of DNA variants in the canine beta-1 adrenergic receptor gene. Res Vet Sci. 95(1):238–240. doi:10.1016/j.rvsc.2013.02.021.

Maran BA, Meurs KM, Lahmers SM, Nelson OL. 2012. Identification of beta-1 adrenergic receptor polymorphisms in cats. Res Vet Sci. 93(1):210–212. doi:10.1016/j.rvsc.2011.05.007.

Martinez M, Modric S, Sharkey M, Troutman L, Walker L, Mealey K. 2008a. The pharmacogenomics of P-glycoprotein and its role in veterinary medicine. J Vet Pharmacol Ther. 31(4):285–300. doi:10.1111/j.1365-2885.2008.00964.x.

Martinez M, Modric S, Sharkey M, Troutman L, Walker L, Mealey K. 2008b. The pharmacogenomics of P-glycoprotein and its role in veterinary medicine. J Vet Pharmacol Ther. 31(4):285–300. doi:10.1111/j.1365-2885.2008.00964.x.

Martinez MN, Court MH, Fink-Gremmels J, Mealey KL. 2018. Population variability in animal health: Influence on dose-exposure-response relationships: Part I: Drug metabolism and transporter systems. J Vet Pharmacol Ther. 41(4):E57–E67. doi:10.1111/jvp.12670.

McEntee M, Silverman JA, Rassnick K, Zgola M, Chan AO, Tau PT, Page RL. 2003. Enhanced bioavailability of oral docetaxel by co-administration of cyclosporin A in dogs and rats. Vet Comp Oncol. 1(2):105–112. doi:10.1046/j.1476-5829.2003.00015.x.

Mealey K. 2016. MDR1 GENE MUTATIONS & DRUG THERAPY. Clinician Brief. Consultado em maio de 2019 Disponível em: https://vcpl.vetmed.wsu.edu/docs/librariesprovider17/default-document-library/ask_-mdr1-gene-mutations-may-2016.pdf?sfvrsn=de7acb38_2

Mealey K. 2018. Pharmacogenomics. In: Veterinary Pharmacology and Therapeutics. Editors: JE Riviere, MG Papich, John Wiley & Sons.

Mealey K. How Should I Treat Dogs & Cats with MDR1 Mutation? https://www.cliniciansbrief.com/article/how-should-i-treat-dogs-cats-mdr1-mutation

Mealey KL. 2004. Therapeutic implications of the MDR-1 gene. J Vet Pharmacol Ther. 27(5):257–264. doi:10.1111/j.1365-2885.2004.00607.x.

Mealey KL. 2006. Adverse Drug Reactions in Herding-Breed Dogs: The Role of P-Glycoprotein. Compendium on Continuing Education for the Practising Veterinarian -North American Edition- 28(1):23-33

Mealey KL. 2008. Canine ABCB1 and macrocyclic lactones: Heartworm prevention and pharmacogenetics. Vet Parasitol. 158(3):215–222. doi:10.1016/j.vetpar.2008.09.009.

Mealey KL. 2012. ABCG2 transporter: therapeutic and physiologic implications in veterinary species: ABCG2 transporter. J Vet Pharmacol Ther. 35:105–112. doi:10.1111/j.1365-2885.2011.01313.x.

Mealey KL, Bentjen SA, Gay JM, Cantor GH. 2001. Ivermectin sensitivity in collies is associated with a deletion mutation of the mdr1 gene: Pharmacogenetics. 11(8):727–733. doi:10.1097/00008571-200111000-00012.

Mealey KL, Burke NS. 2015. Identification of a nonsense mutation in feline ABCB1. J Vet Pharmacol Ther. 38(5):429–433. doi:10.1111/jvp.12212.

Mealey KL, Fidel J, Gay JM, Impellizeri JA, Clifford CA, Bergman PJ. 2008. ABCB1-1Δ Polymorphism Can Predict Hematologic Toxicity in Dogs Treated with Vincristine. J Vet Intern Med. 22(4):996–1000. doi:10.1111/j.1939-1676.2008.0122.x.

Mealey KL, Meurs KM. 2008. Breed distribution of the ABCB1-1Δ (multidrug sensitivity) polymorphism among dogs undergoing ABCB1 genotyping. Sci Rep. 233(6):4.

Mealey KL, Munyard KA, Bentjen SA. 2005. Frequency of the mutant MDR1 allele associated with multidrug sensitivity in a sample of herding breed dogs living in Australia. Vet Parasitol. 131(3–4):193–196. doi:10.1016/j.vetpar.2005.05.004.

Mellstrand T. 1987. Loperamide—an Opiate Receptor Agonist with Gastrointestinal Motility Effects. Scand J Gastroenterol. 22(sup130):65–66. doi:10.3109/00365528709091001.

Merola VM, Eubig PA. 2012. Toxicology of Avermectins and Milbemycins (Macrocylic Lactones) and the Role of P-Glycoprotein in Dogs and Cats. Vet Clin North Am Small Anim Pract. 42(2):313–333. doi:10.1016/j.cvsm.2011.12.005.

Michieli M, Damiani D, Ermacora A, Masolini P, Raspadori D, Visani G, Scheper RJ, Baccarani M. 1999. P-glycoprotein, lung resistance-related protein and multidrug resistance associated protein in de novo acute non-lymphocytic leukaemias: biological and clinical implications. Br J Haematol. 104(2):328–335. doi:10.1046/j.1365-2141.1999.01172.x.

Miller DS. 2002. Xenobiotic export pumps, endothelin signaling, and tubular nephrotoxicants?a case of molecular hijacking. J Biochem Mol Toxicol. 16(3):121–127. doi:10.1002/jbt.10030.

Mise M, Hashizume T, Komuro S. 2008. Characterization of Substrate Specificity of Dog CYP1A2 Using CYP1A2-Deficient and Wild-Type Dog Liver Microsomes. Drug Metab Dispos. 36(9):1903–1908. doi:10.1124/dmd.108.022301.

Mishima H, Kurabayashi M, Tamura C, Sato S, Kuwano H, Saito A. 1975. STRUCTURES OF MILBEMYCIN Bl, B2, and B3. Tetrahedron letters, 10: 711-714.

Muller M, Jansen PL. 1997. Molecular aspects of hepatobiliary transport. Am J Physiol-Gastrointest Liver Physiol. 272(6):G1285–G1303. doi:10.1152/ajpgi.1997.272.6.G1285.

Neff MW, Robertson KR, Wong AK, Safra N, Broman KW, Slatkin M, Mealey KL, Pedersen NC. 2004. Breed distribution and history of canine mdr1-1 , a pharmacogenetic mutation that marks the emergence of breeds from the collie lineage. Proc Natl Acad Sci. 101(32):11725–11730. doi:10.1073/pnas.0402374101.

Elaine A. Ostrander, Anatoly Ruvinsky. 2012. Genetics of the Dog. 2nd Edition CABI.

Parker HG, Dreger DL, Rimbault M, Davis BW, Mullen AB, Carpintero-Ramirez G, Ostrander EA. 2017. Genomic Analyses Reveal the Influence of Geographic Origin, Migration, and Hybridization on Modern Dog Breed Development. Cell Rep. 19(4):697–708. doi:10.1016/j.celrep.2017.03.079.

Parker, H.G.; Kim, L.V.; Sutter, N.B.; Carlson, S.; Lorentzen, T.D.; Malek, T.B.; Johnson, G.S.; DeFrance, H.B.; Ostrander, E.A.; Kruglyak, L. (2004-05-21). "Genetic structure of the purebred domestic dog". Science 304 (5674): 1160. DOI:10.1126/science.1097406

Patel J, Mitra AK. 2001. Strategies to overcome simultaneous P-glycoprotein mediated efflux and CYP3A4 mediated metabolism of drugs. Pharmacogenomics. 2(4):401–415. doi:10.1517/14622416.2.4.401.

Pauli-Magnus C, Kroetz DL. 2004. Functional Implications of Genetic Polymorphisms in the Multidrug Resistance Gene MDR1 (ABCB1). Pharm Res. 21(6):904–913. doi:10.1023/B:PHAM.0000029276.21063.0b.

Pharmacovigilance | European Medicines Agency. 2019 Feb 5. [accessed 2019 Feb 5]. https://www.ema.europa.eu/en/veterinary-regulatory/post-authorisation/pharmacovigilance.

Pirmohamed M. 2001. Pharmacogenetics and Pharmacogenomics_Ginsburg. Br J Clin Pharmacol. 52(4):345–347. doi:10.1046/j.0306-5251.2001.01498.x.

Ramsey I, British Small Animal Veterinary Association. 2017. BSAVA small animal formulary.

Roberts MS, Magnusson BM, Burczynski FJ, Weiss M. 2002. Enterohepatic Circulation: Physiological, Pharmacokinetic and Clinical Implications. Clin Pharmacokinet. 41(10):751–790. doi:10.2165/00003088-200241100-00005.

Roepe PD. 1995. The role of the MDR protein in altered drug translocation across tumor cell membranes. Biochim Biophys Acta BBA - Rev Biomembr. 1241(3):385–405. doi:10.1016/0304-4157(95)00013-5.

Sacco JC, Abouraya M, Motsinger-Reif A, Yale SH, McCarty CA, Trepanier LA. 2012. Evaluation of polymorphisms in the sulfonamide detoxification genes NAT2, CYB5A, and CYB5R3 in patients with sulfonamide hypersensitivity: Pharmacogenet Genomics. 22(10):733–740. doi:10.1097/FPC.0b013e328357a735.

Schinkel AH, Smit JJM, van Tellingen, Beijnen JH, Wagenaar E, van Deemter L. 1994. Disruption of the Mouse m&la P-Glycoprotein Gene Leads to a Deficiency in the Blood-Brain Barrier and to Increased Sensitivity to Drugs. Cell. 1994 May 20;77(4):491-502.
DOI: 10.1016/0092-8674(94)90212-7

Schinkel AH, Wagenaar E, van Deemter L, Mol CA, Borst P. 1995. Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. J Clin Invest. 96(4):1698–1705. doi:10.1172/JCI118214.

Schwarz U, Gramatte T, Krappweis J, Berndt A, Oertel R, Vonrichter O, Kirch W. 1999. Unexpected effect of verapamil on oral bioavailability of the β-blocker talinolol in humans. Clin Pharmacol Ther. 65(3):283–290. doi:10.1016/S0009-9236(99)70107-4.

Seelig A. 1998. A general pattern for substrate recognition by P-glycoprotein. Eur J Biochem. 251(1–2):252–261. doi:10.1046/j.1432-1327.1998.2510252.x.

Stern JA, Reina-Doreste Y, Chdid L, Meurs KM. 2014. Identification of PDE5A:E90K: A Polymorphism in the Canine Phosphodiesterase 5A Gene Affecting Basal cGMP Concentrations of Healthy Dogs. J Vet Intern Med. 28(1):78–83. doi:10.1111/jvim.12256.

Sun H, Frassetto L, Benet LZ. 2006. Effects of renal failure on drug transport and metabolism. Pharmacol Ther. 109(1–2):1–11. doi:10.1016/j.pharmthera.2005.05.010.

Sun J, He Z-G, Cheng G, Wang S-J, Hao X-H, Zou M-J. 2004. Multidrug resistance P-glycoprotein: crucial Published: 2004.01.06 significance in drug disposition and interaction. Med Sci Monit. 2004 Jan;10(1):RA5-14 PMID: 14704647

Swen JJ, Huizinga TW, Gelderblom H, de Vries EGE, Assendelft WJJ, Kirchheiner J, Guchelaar H-J. 2007. Translating Pharmacogenomics: Challenges on the Road to the Clinic. PLoS Med. 4(8):e209. doi:10.1371/journal.pmed.0040209.

Syvänen A-C. 2001. Accessing genetic variation: genotyping single nucleotide polymorphisms. Nat Rev Genet. 2(12):930–942. doi:10.1038/35103535.

Tappin SW, Goodfellow MR, Peters IR, Day MJ, Hall EJ, Mealey KL. 2012. Frequency of the mutant MDR1 allele in dogs in the UK. Vet Rec. 171(3):72.2-72. doi:10.1136/vr.100633.

Tenmizu D, Endo Y, Noguchi K, Kamimura H. 2004. Identification of the novel canine CYP1A2 1117 C>T SNP causing protein deletion. Xenobiotica. 34(9):835–846. doi:10.1080/00498250412331285436.

Trepanier LA. 2004. Idiosyncratic toxicity associated with potentiated sulfonamides in the dog. J Vet Pharmacol Ther. 27(3):129–138. doi:10.1111/j.1365-2885.2004.00576.x.

Trepanier LA, Ray K, Winand NJ, Spielberg SP, Cribb AE. 1997. Cytosolic arylamine n-acetyltransferase (NAT) deficiency in the dog and other canids due to an absence of NAT genes. Biochem Pharmacol. 54(1):73–80. doi:10.1016/S0006-2952(97)00140-8.

Ueda, Kazumitsu, et al. “Expression of a Full-Length CDNA for the Human ``MDR1'' Gene Confers Resistance to Colchicine, Doxorubicin, and Vinblastine.” Proceedings of the National Academy of Sciences of the United States of America, vol. 84, no. 9, 1987, pp. 3004–3008. JSTOR, www.jstor.org/stable/29314.

Vila C. 1999. Phylogenetic relationships, evolution, and genetic diversity of the domestic dog. J Hered. 90(1):71–77. doi:10.1093/jhered/90.1.71.

vonHoldt BM, Pollinger JP, Lohmueller KE, Han E, Parker HG, Quignon P, Degenhardt JD, Boyko AR, Earl DA, Auton A, et al. 2010. Genome-wide SNP and haplotype analyses reveal a rich history underlying dog domestication. Nature. 464(7290):898–902. doi:10.1038/nature08837.

Wandel C, Kim R, Wood M, Wood A. 2002. Interaction of Morphine, Fentanyl, Sufentanil, Alfentanil, and Loperamide with the Efflux Drug Transporter P-glycoprotein: Anesthesiology. 96(4):913–920. doi:10.1097/00000542-200204000-00019.

West IC. 1990 Feb. What determines the substrate specificity of the multi-drug-resistance pump? Trends Biochem Sci. 1990 Feb; 15(2):42-6 DOI: 10.1016/0968-0004(90)90171-7

Westphal K. 2000. Oral bioavailability of digoxin is enhanced by talinolol: Evidence for involvement of intestinal P-glycoprotein. Clin Pharmacol Ther. 68(1):6–12. doi:10.1067/mcp.2000.107579.

Woodward K. 2009. Veterinary Pharmacovigilance: Adverse Reactions to Veterinary Medicinal Products. John Wiley & Sons.

Yamaguchi H, Yano I, Saito H, Inui K. 2002. Pharmacokinetic Role of P-Glycoprotein in Oral Bioavailability and Intestinal Secretion of Grepafloxacin in Vivo. J Pharmacol Exp Ther. 300(3):1063–1069. doi:10.1124/jpet.300.3.1063.

Yoshimura A, Kuwazuru Y, Sumizawa T, Ichikawa M, Ikeda S, Uda T, Akimyama S. 1989. Cytoplasmic Orientation and Two-domain Structure of the Multidrug Transporter, P-glycoprotein, Demonstrated with Sequence-specific Antibodies. J Biol Chem. 1989 Sep 25;264(27):16282-91

Zhu M, Yancy HF, Deaver C, Jones YL, Myers MJ. 2016. Loperamide-induced expression of immune and inflammatory genes in Collies associated with ivermectin sensitivity. J Vet Pharmacol Ther. 39(2):131–137. doi:10.1111/jvp.12268.

V. Attachments

Attachment 1 - FCI Breed Group

GROUP	DESIGNATION	BREEDS
1	Sheepdogs and Cattledogs (except Swiss Cattledog)	Australian Kelpie, Belgian Shepherd Dog, Schipperke, Croatian Shepherd dog, Berger de Beauce, Briard, Long-haired Pyrenean Sheepdog, Picardy Sheepdog, Pyrenean Sheepdog, German Shepherd dog, Bearded Collie, Border Collie, Collie Rough, Collie Smooth, Olde English Sheepdog, Shetland Sheepdog, Welsh Corgi, Komondor Kuvasz, Mudi, Puli, Pumi, Bergamasco Shepherd dog, Maremma and the Abruzzes Sheepdog, Polish Lowland Sheepdog, Tatra Shepherd dog, Portuguese Sheepdog (Cão da Serra de Aires), Romanian Carpathian Shepherdog, Romanian Mioritic Shepherd Dog, South Russian Shepherd dog, Czechoslovakian Wolfdog, Slovakian Chuvach, Catalan Sheepdog, Majorca Sheepdog, White Swiss Shepherd dog, Dutch Schapendoes, Dutch Shepherd dog, Saarloos Wolfhond, Australian Shepherd, Australian Cattle dog, Bouvier des Ardennes, Bouvier des Flandres
2	Pinscher and Schnauzer – Molossoind and Swiss Mountain and Cattledogs	Australian Pincher, Danish-Swedish Farmdog, Affenpincher, Dobermann, German Pincher, Miniature Pincher, Gient Schnauzer, Miniature Schnauzer, Schnauzer, Dutch Smoushond, Russian Black Terrier, Dogo Argentino, Fila Brasileiro, Shar Pei, Broholmer, Dogue de Bordeaux, Boxer, Great Dane, Rottweiler, Bulldog, Bullmastiff, Mastiff, Italian Cane Corso, Neapolitan Mastiff, Tosa, Saint Miguel Cattle Dog (Cão de Fila de São Miguel), Majorca Mastiff, Presa Canario, Cimarrón Uruguayo, Bosnian and

		Herzegovinian – Criatian Shepherd dog, Newfoundland, Pyrenean Mountain dog, Hovawart, Leonberger, Landseer, Atlas Mountain dog (Aidi), Yugoslavian Shepherd dog – Sharplanina, Castro Laboreiro Dog (Cão de Castro Laboreiro), Estrela Mountain Dog (cão da Serra da Estrela), Rafeiro of Alentejo (Rafeiro do Alentejo), Romanian Bucovina Sheperd, Caucasian Shepherd dog, Central Asia Shepherd dog, Karst Shepherd dog, Pyrenean Mastiff, Spanish Mastiff, St. Bernard, Tibetan Mastiff, Kangal Shepherd dog, Appenzell Cattle Dog, Bernese Mountain dog, Entlebuch Cattledog, Great Swiss Mountain dog
3	Terriers	Brazilian Terrier, German Hunting Terrier, Airedale Terrier, Bedlington Terrier, Border Terrier, Fox Terrier Lakeland Terrier, Manchester Terrier, Parson Russell Terrier, Welsh Terrier, Irish Glen Imaal Terrier, Irish Soft Coated Wheaten Terrier, Irish Terrier, Kerry Blue Terrier, Australian Terrier, Ceský Terrier, Cairn Terrier, Dandie Dionmont Terrier, Jack Russell Terrier, Norfolk Terrier, Norwich Terrier, Scottish Terrier, Sealyham Terrier, Skye Terrier, West Highland White Terrier, Japanese Terrier, Bull Terrier, Miniature Bull Terrier, Staffordshire Bull Terrier, American Staffordshire Terrier, Australian Silky Terrier, English Toy Terrier, Yorkshire Terrier
4	Dachshunds	Dachshund
5	Spitz and primitive types	Canadian Eskimo dog, Greenland dog, Samoyed, Alaskan Malamute, Siberian Husky, Finnish Spitz, Karelian Bear dog, Norwegian Elkhound, Norwegian Lundehund, East Siberian Laika, Russian-European Laika, West Siberian Laika, Jamthund, Norrbottenspitz, Finnish Lapponian dog, Lapponian Herder, Iceland

		Sheepdog, Norwegian Buhund, Swedish Lapphund, Swedish Vallhund, German Spitz, Italian Volpino, Chow Chow, Eurasian, Akita, American Akita, Hokkaido, Japanese Spitz, Kai, Kishu, Shiba, Shikoku, Korea Jindo dog, Basenji, Canaan dog, Pharaoh Hound, Xoloitzcuintle, Peruvian Hairless dog, Cirneco Dell'Etna, Podengo Portuguese Warren Hound-Portuguese Podengo, Canarian Warren Hound, Ibizan Podenco, Taiwan dog, Thai Ridgeback dog
6	Scent hounds and related breeds	Bloodhound, Billy, French Tricolour Hound, French White & Black Hound, French with and Orange Hound, Gascon Saintongeois, Grand Griffon Vendeen, Great Anglo-french Tricolour Hound, Great Anglo-french white and black Hound, Great Gascony Blue, English Foxhound, Otterhound, Polish Hound, American Foxhound, Black and Tan Coonhound, Australian Black and Tan Hound, Coarse-Haired Styrian Hound, Tyrolean Hound, Bosnian Broken-Haired Hound, Istrian Short-Haired Hound, Istrian Wire-haired Hound, Posavatz Hound, Finnish Hound, Ariegeois, Artois Hound, Beagle Harrier, Blue Gascony Griffon, Briquet Griffon Vendeen, Fawn Brittany Griffon, Griffon Nivernais, Medium-sized Anglo-french Hound, Poitevin, Porcelain, Small Blue Gascony, Harrier, Hellenic Hound, Hungarian Hound – Transylvanian Scent Hound, Italian Rough-haired Segugio, Italian Short-haired Segugio, Montenegrin Mountain Hound, Halden Hound, Hygen Hound, Norwegian Hound, Polish Hunting dog, Serbian Tricolour Hound, Slovakian Hound, Spanish Hound, Hamiltonstovare, Schillerstovare, Smalandsstovare, Swiss Hound, Basset Fauve de Bretagne, Blue Gascony Basset, Grand

		Basset Griffon Vendeen, Norman Artesien Basset, Petit Basset Griffon Vendeen, German Hound, Westphalian Dachsbracke, Basset Hound, Beagle, Drever, Small Swiss Hound, Alpine Dachsbracke, Bavarian Mountain Scent Hound, Hanoverian Scent Hound, Dalmatian, Rhodesian Ridgeback
7	Pointing dogs	Ariege Pointing dog, Auvergne Pointer, Bourbonnais Point dog, French Pointing dog – Gascogne type, French Pointing Dog – Pyrenean type, Saint German Pointer, Deutsch Stichelhaar, German Short-haired Pointing dog, German wire-haired Pointing dog, Pudelpointer, Weimaraner, Hungarian Short-haired Pointer, Hungarian wire-haired Pointer, Italian Pointing dog, Portuguese Pointing dog, Wirehaired Slovakian Pointer, Burgos Pointing dog, Blue Picardy Spaniel, Brittany Spaniel, French Spaniel, Picardy Spaniel, Pont-Audemer Spaniel, Deutsch Langhaar, Kleiner Munsterlander, Large Munsterlander, Drentsche Partridge dog, Stabijhoun, Bohemian Wire-haired Pointing Griffon, Wire-haired Pointing Griffon Korthals, Italian Spinone, English Pointer, English Setter, Gordon Setter, Irish Red and White Setter, Irish Setter
8	Retrievers – Flushing Dogs – Waterdogs	Nova Scotia Duck Tolling Retriever, Curly Coated Retriever, Flat Coated Retriever, Golden Retriever, Labrador Retriever, Chesapeake Bay Retriever, German Spaniel, Clumber Spaniel, English Cocker Spaniel, English Springer Spaniel, Field Spaniel, Sussex Spaniel, Welsh Springer Spaniel, Nederlandse Kooikerhondje, American Cocker Spaniel, French Water Dog, Irish Water Spaniel, Romagna Water Dog,

		Portuguese Water Dog, Spanish Water Dog, Frisian Water Dog, American Water Spaniel
9	Companion and Toy Dogs	Bichon Frise, Maltese, Havanese, Bolognese, Coton de Tulear, Little Lion Dog, Caniche (Poodle), Griffon Belge, Griffon Bruxellois, Petit Brabançon, Chinese Crested Dog, Lhasa Apso, Shih Tzu, Tibetan Spaniel, Tibetan Terrier, Chihuahua, Cavalier King Charles Spaniel, King Charles Spaniel, Pekingese, Japanese Chin, Continental Toy Spaniel, Russian Toy, Kromfohrlander, Pug, French Bulldog, Boston Terrier
10	Sighthounds	Afghan Hound, Saluki, Russian Hunting Sighthound, Deerhound, Irish Wolfhound, Greyhound, Whippet, Hungarian Greyhound, Italian Sighthound, Azawakh, Sloughi, Polish Greyhound, Spanish Greyhound