UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

VETERINARY PHARMACOVIGILANCE, FROM REGULATION TO SCIENTIFIC EXPLANATION. CASE STUDIES OF CANINE MDR1 MUTATION

RITA ISABEL COSTA BENTO

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I dedicate this work to Pedro Marques…you are always there!

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À professora Berta, mais do que uma professora, uma amiga. Obrigada por toda a sua dedicação. Espero que continue a inspirar os seus alunos como me inspirou a mim.

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"If a man knows what harbor he seeks, any wind is the right wind. If one does not know to which port one is sailing, no wind is favorable"(Lucius Annaeus Seneca).

Abstract

VETERINARY PHARMACOVIGILANCE, FROM REGULATION TO SCIENTIFIC EXPLANATION. CASE STUDIES OF CANINE MDR1 MUTATION

Veterinary pharmacovigilance is the science and activities related to the detection, assessment, understanding and prevention of adverse effects or any other medicine-related problem associated to veterinary medicinal products. It seems that some breeds are more sensitive than others to this type of events and understanding this sensitivity is permitted by the pharmacogenetics discipline.

Nowadays, there is a national legislation for all countries of EU to implement a veterinary pharmacovigilance system. However, situations and philosophy vary from one country to another and, for example, in France the system works very differently.

The Collie breed is known for having a special sensitivity to the ivermectin drug and this will be taken as an example to explain the existence of genetic particularities among breeds, such as, in this case, a mutation in the multidrug resistance gene 1 that encodes a large transmembrane protein cell, namely P-glycoprotein. Using data from the Sentinel-Vet software, it was investigated the existence of a superior number of adverse drug reactions reported to CPVL (Veterinary Pharmacovigilance Center of Lyon) related with breeds which have present the referent mutation within their population. It was also made a study with an innovative treatment based on intravenous lipid emulsions, applied in 7 cases after intoxication with avermectins.

Keywords: pharmacovigilance, pharmacogenetics, breed sensitivities to medicines, multidrug resistance gene 1, P-glycoprotein, intravenous lipid emulsions.

Resumo

FARMACOVIGILÂNCIA VETERINÁRIA, DA REGULAMENTAÇÃO À SUA APLICAÇÃO CIÊNTIFICA. ESTUDO DE CASO DA MUTAÇÃO CANINA MDR1.

A farmacovigilância veterinária é definida como uma ciência que envolve as atividades relacionadas com a deteção, avaliação, compreensão e prevenção de efeitos adversos ou quaisquer problemas relacionados com o uso de medicamentos veterinários. Nos diferentes indivíduos da mesma espécie parecem existir raças mais sensíveis que outras a este tipo de eventos, e a compreensão desta sensibilidade é abordada pela farmacogenética.

Atualmente existe uma legislação nacional para a implementação de um sistema de farmacovigilância em todos os países da EU. Contudo, este pode variar conforme a filosofia do país.

A raça canina Collie é conhecida por ter uma sensibilidade especial à ivermectina, e este facto é tomado como exemplificativo para a existência de particularidades genéticas dentro de determinadas raças, tais como, neste caso, uma mutação no gene da multiresistência 1 que codifica uma grande proteína transmembranar, a glicoproteína P. Através da análise dos dados do programa Sentinel-Vet foi investigada a existência de um número superior de reações adversas, reportadas ao CPVL (Centro de Farmacovigilância Veterinária de Lyon), relacionadas com as raças que têm presente na sua população a mutação referida. Foi realizado, igualmente, um estudo para o tratamento de intoxicações através do uso de emulsificações lipídicas intravenosas, tendo sido analisados 7 casos após intoxicação por avermectinas.

Palavras-chave: farmacovigilância, farmacogenética, sensibilidades raciais a medicamentos, gene da multiresistência 1, glicoproteína P, emulsões lipídicas intravenosas.

Brief description of the activities carried out in the curricular internship

The internship which served as base to write this dissertation was performed in the area of Pharmacology and Toxicology, more specifically, on Veterinary Pharmacovigilance. Corresponding to the sixth year of a Masters in Veterinary Medicine, by the *Faculdade de Medicina Veterinária da Universidade Técnica de Lisboa*, it was divided into two parts:

1) The first part, between the 3rd of October and the 7th February 2011/2012, took place under the Erasmus program in Lyon, France. The *École Nationale Vétérinaire de Lyon* allowed the realization of the internship on CPVL (Veterinary Pharmacovigilance Centre of Lyon) and on CNITV (National Centre for Veterinary Toxicological Information) under the supervision of Dr. Xavier Pineau. CPVL collects reports of suspected adverse reactions to veterinary medicinal products in animals and humans, provided by all health professionals. It has an assessment cases mission, improvement of knowledge and transmission of this information to the National Competent Authority, the *Agence Nationale du Médicament Vétérinaire* (ANMV or French Agency for Veterinary Medicinal Products) - frame of the new French Agency for Food, Environmental and Occupational Health Safety. This center also provides training of pharmacovigilance for all professionals concerned. This center, founded in 1976, benefits from the 36 years of pharmacovigilance experience realized by CNITV and has represented the French concept of veterinary pharmacovigilance since 2001. This concept differs from other European countries, where the notifications have an administrative character. The center runs 24 hours a day. At CPVL/CNITV the system works as mutualism association giving diagnostics, advice treatments and prognostics to the caller. In turn, the center has the best database of pharmacovigilance cases.

   The intern participated in all these areas, including working with the Sentinel-Vet software, cases assessment and telephone support to several cases reported to CPVL. This active participation allows a better understanding of the reciprocal French pharmacovigilance system. Additionally, the intern made a guided visit to the pharmaceutical company Merial in Lyon.

2) The second part was performed at *Direção Geral de Alimentação e Veterinária* in the department of *Medicamentos veterinários, produtos de uso veterinário e biocidas de uso veterinário* on *Sistema Nacional de Farmacovigilância Veterinária* (SNFV or National Veterinary pharmacovigilance system), under the supervision of Dr. Henrique Ramos da

Costa. The internship lasted approximately 2 months (16 February to 27 April) and had the purpose to contact with the Portuguese reality in terms of pharmacovigilance, as well as its legal basis. Here, the student had an active role in the valuation and assessment of reports of suspected adverse reactions and in the evaluation of Periodic Safety Update Reports, submitted by marketing authorizations holders, for medicinal veterinary products with national procedures. The student also participated in decision-making about possible risk management measures (resulting from the reporting of adverse events), contact with Eudravigilance software and contribution in other activities related to the processes of regulation of veterinary medicines, such as: publicity, quality defects and licensing procedures (wholesale distribution and retail sale) with Dr. Maria da Luz Grencho. The official internship report is in annexe 1 (Portuguese).

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ABC: ATP-binding cassette.

ADR: adverse drug reaction.

ANMV: Agence nationale du médicament vétérinaire.

ANSES: Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail.

CNITV: Centre National d'Informations Toxicologiques Vétérinaires.

CPVL: Centre de Pharmacovigilance Vétérinaire de Lyon.

EEA: European Economic Area.

EFTA: European Free Trade Association.

EU: European Union.

EVVet: Eudravigilance- Vet.

ILE: Intravenous Lipid Emulsion.

MA: Marketing authorization.

MDR: multidrug resistance.

MDR1: multidrug resistance 1.

MIA: Market Introduction Authorization.

MS: Member State.

NCA: National Competent Authorities.

Pgp: P-glycoprotein.

PSUR: Periodic safety updates report.

SAR: Suspected adverse reaction.

SPC: Summary of Product Characteristics.

VMP: Veterinary medicine product.

1 Introduction

Veterinary medicine has the unique challenge of having to treat a large number of domestic animal species, namely mammals, birds, reptiles, amphibians, fishes and others.

A great number of doctor appointments are related with toxicology and part of them is due to molecules commonly used. However, some breeds seem more sensitive than others to this type of events and understanding this sensitivity, previously described as idiosyncratic, is permitted by the pharmacogenetics discipline. Veterinary pharmacogenetics is a new branch of veterinary science that has the purpose to identify the genetic variations (polymorphisms) responsible by differences in the drug response of individuals within a given species (Cunningham F., Elliott J. & Lees P. 2010).

A breed is defined as a group of animals having common ancestry and certain distinguishable characteristics developed by artificial selection and maintained by controlled propagation (Licinio J. & Wong M., 2002). The first animal species to be domesticated and subsequently selected bred for thousands of years were dogs, which leads into a wide variety of more than 400 breeds worldwide with differing anatomical, physiological, and behavioural traits (Cunningham *et al*., 2010).

The fixation of phenotypic appearance and the mating of closely related individuals have resulted in breed specific disease patterns and great variations in life expectancy. Therefore, with this large genetic diversity within the canine species it is not surprising that there are both metabolic and physiologic idiosyncrasies that can influence not only the propensity for certain disease conditions, but also drug pharmacokinetics, pharmacodynamics and the characteristics of responses to xenobiotics (Fleischer S., Sharkey M., Mealey K., Ostrander E. & Martinez M., 2008). Studies have shown that the occurrence of adverse drug reactions (ADRs) is due to many factors including genetics ones (Nicholas T., *et al* 2009).

In humans only 5% to 10% of genetic variation has been shown to be associated with populations or ethnicities, although in dogs 27% of genetic variation is associated with differences in breeds (Fleischer *et al*, 2008). The existence of a genetic variation among dog breeds results in a risk of breed-related differences in the effectiveness and toxicological responses to drugs. Therefore, it is possible to consider that the existence of a pharmacovigilance system is indispensable for detecting these genetic and hereditary different sensitivities, interspecies and even within them, to some drugs.

The following thesis, in its first part, will conduct a literature review about the importance of veterinary pharmacovigilance in the detection of certain sensitivities to xenobiotics within a given species. Collies, and related breeds, will be taken as an example since they have present

among their population some individuals with a mutation in the multidrug resistance gene 1 (MDR1) that encodes a large transmembrane protein cell, P-glycoprotein (Pgp), that can conduct to adverse reactions to some drugs.

Furthermore, in one second and practical part, it will be investigated the existence, or not, of a superior number of adverse drug reactions reported to CPVL related with these breeds. These data was obtained from the Sentinel-Vet software and the three substances of study are ivermectin, loperamide and emodepside, since they are Pgp subtracts. As well, it is required that CPVL specialists advise veterinarians, who are contacted by phone, for the necessary follow-up after intoxication. Taking this into account an innovative treatment based in intravenous lipid emulsions (ILE) is presented by the description of 7 cases which used ILE as an antidote after overdose with lipophilic drugs.

2 Literature Review

2.1 Veterinary Pharmacovigilance

When a new veterinary medicinal product is put into the market, adverse drug reactions may become apparent. This can be seen in the treated animal patients, in exposed users or as adverse effects on the environment. Moreover, they might be evident for excess of drug residues in food with animal origin. Consequently, legislation and regulatory approaches were established across the globe to address these issues and to guarantee that the constant safety of these products can be monitored and, where necessary, that regulatory actions can be followed to solve any concerns. The single term "pharmacovigilance" can cover all of these issues (Woodward K., 2009) and the Veterinary pharmacovigilance has shown a remarkable development in recent years (Keck G. & Ibrahim C., 2001).

2.1.1 Definition of veterinary pharmacovigilance

Defined by the World Health Organization, pharmacovigilance is the science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other medicine-related problem. This principle also applies to medicinal products for veterinary use (VMPs) (EMEA, 1996).

2.1.2 Importance of veterinary pharmacovigilance

Before a company can place a VMP in the market there is a requirement, under European Union (EU) or national legislation, for a Marketing Authorisation (MA). This MA is just approved after several laboratory and then preclinical and clinical studies. Obvious reasons of time, cost, and studies rationalization, lead to studies performed directly on target species applying a small number of animals. However, on the market, the medicine will be used on an infinitive number of animals and several different clinical conditions. It will be used in diverse breeds with their particular metabolic characteristics or different sensitivities, on variable grades pathologies, in animals with medical history or other drugs therapy. Furthermore, the drug will not be administered just by professionals but also by animal's owners. As a result, only the daily practice conditions can lead to the drug safety and pharmacovigilance should allow the collection of each SAR in animals or, when in contact with treated animals, in humans.

Subsequent to the collection of cases, each of them should be performed, evaluated and recorded in a database. The analysis of these cases can provide a better understanding of

possible side effects, their frequency, risk factors occurrence and prognosis relating to a specific specie or breed (Woodward K., 2009).

Some species have a very low therapeutic variation, as it is the case of many "new pets" and the experience of all is then necessary to allow the prescription of the most appropriate, use and dose, to generate the less possible risk for the treated animal. Thus, even with "of-label use", every incident or accident that may have medication origin should be constituted as pharmacovigilance case (Pineau X., 2012).

Pharmacovigilance has gradually gained new fields of activity covering not only clinical safety but also other aspects of post-authorisation surveillance. These include: lack of expected efficacy and misuses of a veterinary medicine, human reactions to veterinary medicines, epidemic-surveillance of resistance, potential environmental problems and reported violations of approved residue limits (EMEA, 1996).

2.1.3 Regulation of pharmaceutical products

Any pharmaceutical product needs a MA, and to be sold in one or more EU countries needs to follow one of the legal options in order to be registered:

2.1.3.1 Centrally Authorised Product

A product for which the MA is granted, in agreement with the Directive 2001/83/EC and 2004/28/EC, regulation (EC) No 726/2004, by the European Commission. The grant is valid in all EU and "European Free Trade Association States" (EFTA) (Iceland, Liechtenstein and Norway).

2.1.3.2 Decentralised procedure

A procedure used in order to obtain MA in several Member States, where the VMP has not yet received a MA in any Member State at the time of application (Directive 2001/83/EC).

2.1.3.3 Mutual recognition procedure

A procedure whereby concerned MSs recognize the MA already decided by a reference MS. The placing on the market of the product in their national territory, as outlined by Directive 2001/82/EC and 2004/28/EC is authorized.

As well as these three ways, there are national authorisations (National procedure) which allows a product to be marketed in a particular member state or states granting them. This can be useful to apply for authorisation under the Mutual Recognition Procedure. Wherever the single national MA is, it will become the country which assumes responsibility for monitoring and safety assessment for that particular product (Barton J., 2009).

2.1.4 Legal Framework for Pharmacovigilance

The legal basis for pharmacovigilance of VMP in the EU is presented in Directive 2001/82/EC2, as last amended by Directive 2004/28/EC3, Regulation (EC) No 726/2004. For all VMPs authorised in the EU, counting those authorised before 1 January 1995 and whatever procedure was used for their authorization, must follow pharmacovigilance regulations.

The duties of the National Competent Authorities (NCA) of MS to govern a system for pharmacovigilance is, in order, to collect, collate and evaluate information about SARs. The obligation of MAHs for the creation and maintenance of a pharmacovigilance system is described in Article 74 of Directive 2001/82/EC and Article 48 of Regulation (EC) No 726/2004. NCA and marketing authorization holders (MAH) must share, among them, all important information in order to allow all parties involved in pharmacovigilance activities to assume their obligations and responsibilities.

The European Medicines Agency (EMA), subsequently stated as Agency, and the NCAs collaborate to incessantly develop pharmacovigilance systems able to achieve public health protection for all VMPs, regardless of routes of authorisation, including the use of collaborative approaches, to maximise use of resources available within the EU, in agreement with Article 53 of Regulation (EC) No 726/2004. To operate this system is necessary an intensive exchange of information between the MAH, the NCA, the Agency and the European Commission, as well as procedures to prevent duplication, maintain confidentiality and ensure the quality of the systems and data.

An "Agreement of the European Economic Area" (EEA) was done within some countries, adopting the complete *acquis communautaire* on medicinal products, and they are consequently parties to the EU procedures, as Iceland, Liechtenstein and Norway (Woodward K., 2009).

2.1.5 Harmonization of the causality assessment for ADRs

The regulatory schemes for spontaneous adverse reports of human or veterinary medicines include a requirement to assign causality and to report unexpected adverse drug reactions. Consequently, there is a necessity to identify if a particular product was responsible for a particular adverse reaction and, if so, whether that reaction was unexpected, or otherwise, listed on the label and product literature. In the Volume 9B (EMA, 2011) important issues of causality and the application of the ABON system were addressed (Woodward K., 2009). The classification of causality of adverse reactions is resumed on the table 1.

However, it should be noted that this classification is not fixed since the drug knowledge kept bound with scientific knowledge (molecules, pathogenic mechanisms or metabolic), clinical cases publications and registration of similar notification in pharmacovigilance system database (Pineau X., 2012).

Table 1: Criteria for causality assessment of an ADR (adapted from: Woodward K., 2009).

Classification	Criteria
"A" - probable	All of the following: • Reasonable association in time between drug administration and onset and duration of the adverse effect; • Positive challenge/dechallenge; • Clinical or pathological phenomena must be reliable with the adverse reaction, or reasonable, according the known pharmacology and toxicology; • No similarly plausible explanation. Simultaneous use of other drugs or intercurrent disease, exclusion of other causes; • Where any of the above cannot be satisfied, consider B, N or O or O1.
"B" - possible	• Drug causality is one of other possible or plausible causes; • Data do not meet inclusion criteria for A.
"O"-Unclassifiable	• Insufficient data to make any conclusions.
"O1" – Inconclusive	• Other factors prevented a conclusion. However, an association with product treatment cannot be eliminated.
"N" - Unlikely	• Unlikely to be product related.

In causality assessment the following factors should be taken into account:

1) Associative connection: on time (including dechallenge and rechallenge following repeated administration) and on anatomic sites.
2) Pharmacological explanation, blood levels and previous knowledge of the drug.
3) Presence of clinical characteristics or pathological phenomena.
4) Exclusion of other causes, completeness and reliability of data in case reports.
5) Quantitative measurement of the contribution degree of a drug to the reaction development (dose-effect relationship).

2.1.6 EudraVigilance: the European common system for monitoring veterinary drugs safety

EudraVigilance (EVVet) is a central EU database for electronic reporting of suspect adverse reactions to veterinary medicines, used by the national competent authorities. There are 32 competent authorities registered with a total of 217 different users, and Luxemburg, Maltese and Romanian authorities are requested to progress with the final registration to EVVet production system. In relation to pharmaceutical industry, 203 organisations are registered (MAHs and third parties) with a total of 364 different users. This software allows an electronic exchange of SAR reports (referred to as Individual Case Safety Reports) between the EMA, NCAs, MAHs and sponsors of clinical trials in the EEA. Furthermore, this software facilitates the process of risk analysis at different levels including aspects of risk detection, risk assessment, risk minimisation and risk communication. The reporting obligations of the various stakeholders are defined in the Community legislation, particularly, in the Directive 2001/83/EC, Regulation (EC) No 726/2004 (Woodward K., 2005a).

2.1.7 Veterinary pharmacovigilance in France: a different approach

National legislation exists now in all EU to implement a veterinary pharmacovigilance system. Though, the situation and philosophy vary from one country to another (Keck G. & Ibrahim C., 2001). In Portugal, the veterinary pharmacovigilance system rests only at authority's level, in the named *Direção Geral de Alimentação e Veterinária*, corresponding to the Portuguese National Authority for Animal Health. However, in France a different system exists.

2.1.7.1 History of French pharmacovigilance

In France, serious cases of ADRs in human highlighted the need for a reliable pharmacovigilance system to detect the earliest possible unintended effects of medicinal products and to enable authorities to respond immediately. The first Symposium on Veterinary Pharmacovigilance was held at the veterinary School of Lyon, on April 24-25, 1990, organized by the CNITV, under the auspices of French Ministry of Agriculture and the Ministry of Health and Welfare (Keck G., 1992).

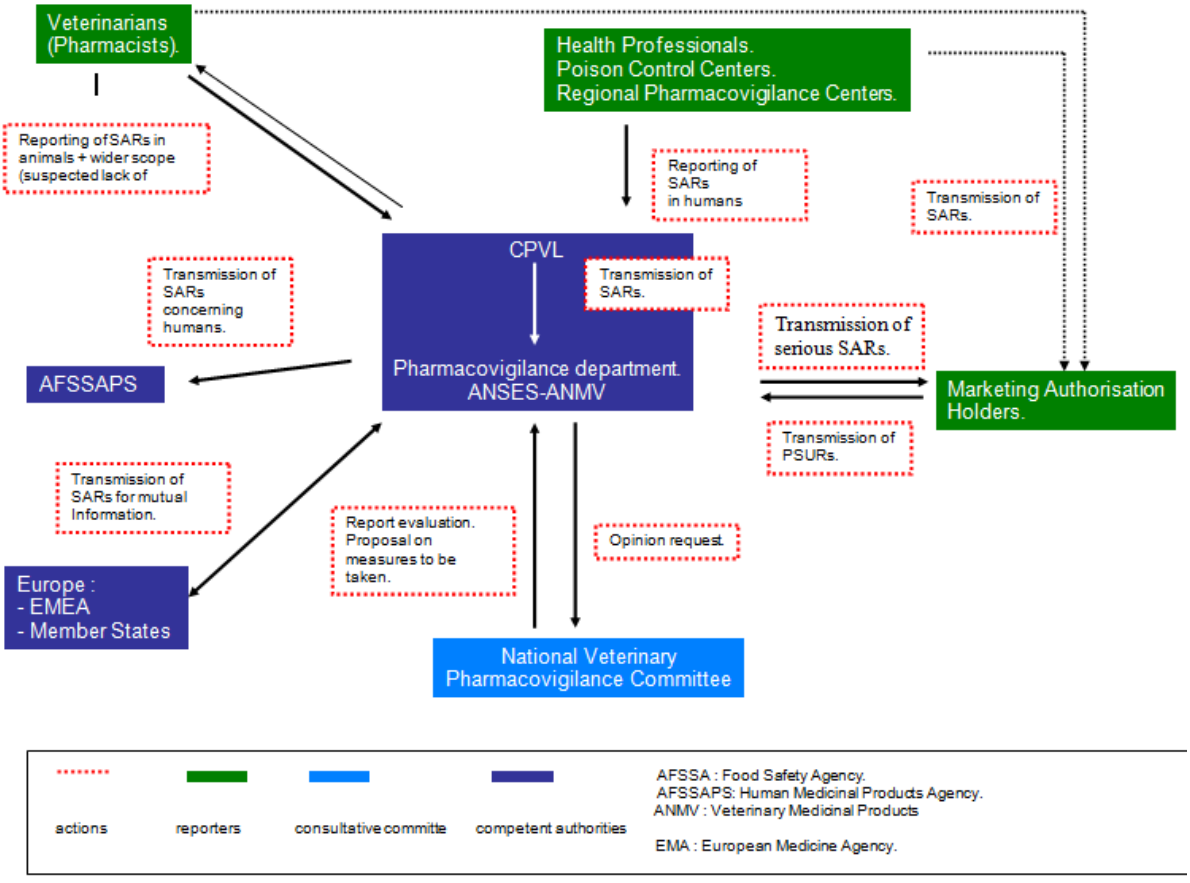
The legal basis of French veterinary pharmacovigilance was established in 1999, and the pharmacovigilance scheme has been fully operational since January 2002. The *Agence Nationale du Médicament Vétérinaire* (ANMV or French Agency for Veterinary Medicinal Products) is responsible for organising and managing the pharmacovigilance scheme under the framework of French Agency for Food, Environmental and Occupational Health Safety

(ANSES). In 1999, occurred a transposition of European regulations and an adaptation to the Human French pharmacovigilance legislation (Pineau X., 2012).

2.1.7.2 French concept of veterinary pharmacovigilance and elements involved

Here, the pharmacovigilance system is an "interactive" concept with the unique center of pharmacovigilance in Europe, the CPVL, located in the Veterinary School of Lyon (Woodward K., 2009). Most of the time (95%), reports are made by telephone and information is exchanged directly between the reporter and trained pharmacovigilance personnel. The center gives an immediate answer to questions raised by the caller (information related with the first evaluation of clinical plausibility of drug action, relevant therapy and prognosis) while the center registers the notification report (Keck, G., 1992). All information is recorded in a standard computerised form, the Sentinel-Vet software, and data are regularly evaluated by the ANSES-ANMV, which recommends to the authorities the steps to be taken in order to minimise the risks of adverse effects (Keck G. & Ibrahim C., 2001).

Figure 1: The veterinary pharmacovigilance centres in France and their interrelationships with other organisations involved (adapted from: Pineau X., 2012).



2.1.7.3 Notification, the basis of French pharmacovigilance system

Veterinary practitioners are the basis of veterinary pharmacovigilance: they provide 89.6% of the notifications received by the CPVL and 67.33% of total recorded statements to ANMV, well ahead of doctors and pharmacists with 4.71% and 0.23% respectively. To notify, the French veterinary practitioners have at their disposal several models and they can choose the one that best suits their daily practice like telephone, written notification in a defined declaration form and by email. This center collects and evaluates all notifications, except from pharmaceutical companies, and is supported by the CNITV, which ensures a permanently 24 hours per day and 7 days per week of operation, through a common telephone access. To transform a case into a pharmacovigilance notification it is necessary: an identified "notifier", at least one species and one drug, and clinical signs correctly described. If anything is missing the file will be closed without further developments (Pineau X., 2012).

2.1.7.4 The role of pharmaceutical companies localized in France

The pharmaceutical companies in France have their own pharmacovigilance and notification system and it is also controlled by ANMV. Information is collected from the field reported by the veterinary practitioners, sometimes with an indirect reason as claims and diagnostic support. Companies are legally obliged to inform ANMV of serious adverse reactions within 15 days, and non-serious adverse reactions are included in PSURs. These are fundamental and complementary parts because they relate adverse effects that fail to CPVL or ANMV.

2.1.7.5 Monitoring results of VMP by CPVL

In 2010, there were 2 377 reports of adverse reactions recorded by the CPVL. Plus, 175 inquiries and 424 cases that remained asymptomatic.

The domestic carnivores represent 69% of all pharmacovigilance notifications recorded in 2010 (figure 2). This fact is logical because they are the most supervised by the owners and with more access to a veterinary clinic. As far as the canine species is concerned, the accidental ingestions of drugs are also common. In dogs, the most common notified products are the external antiparasitics associated to an overdose or accidental ingestion. Following, are the internal antiparasitics and endectocides (figure 3).

Figure 2: Percentage of notification reports relatively to the type of specie concerned- CPVL, 2010 (adapted from: Pineau X., 2012).

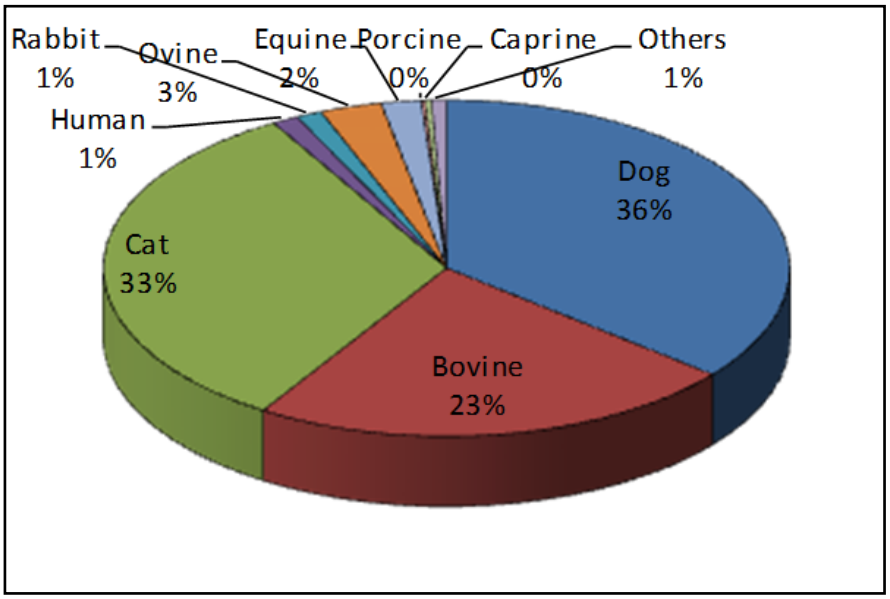
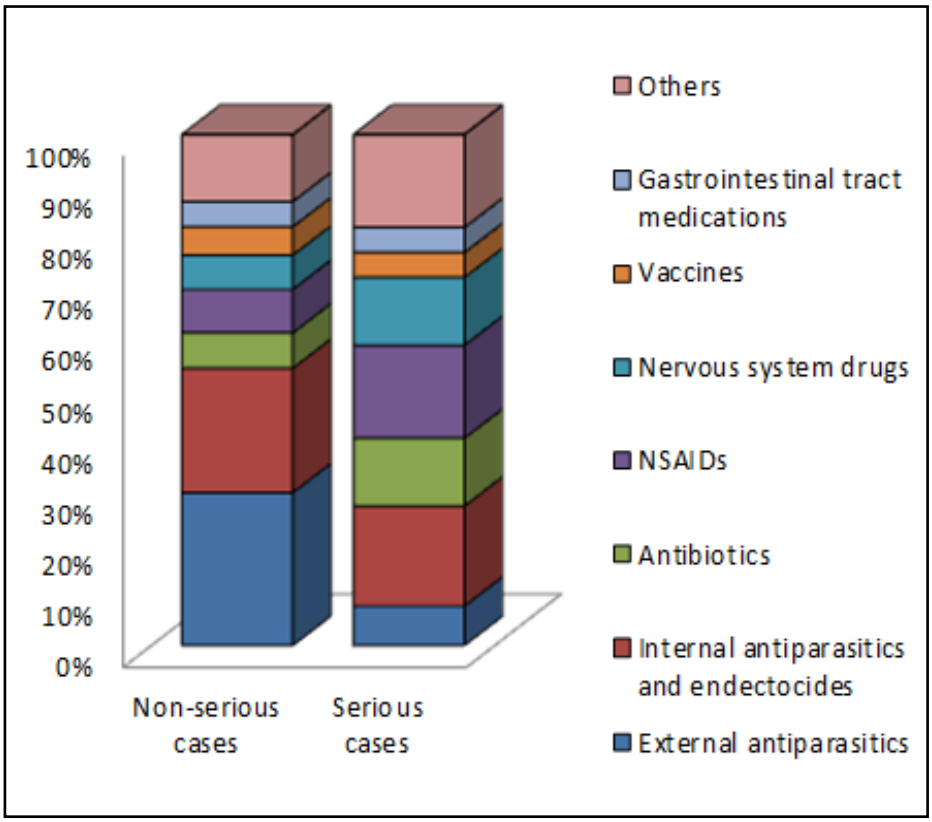


Figure 3: Distribution of reported cases concerning dogs by therapeutic classes – CPVL, 2010 (adapted from: Pineau X., 2012).



2.2 Veterinary Pharmacogenetics

Briefly, Veterinary pharmacogenetics can be defined as the result of converging pharmacology and genetics, which deals with genetically determined responses to drugs. Despite the lack of genetic information in veterinary medicine, specific differences within breeds, named genetic subgroups, in response to endogenous and exogenous substances have been reported. The high genetic variety leads to metabolic and physiologic idiosyncrasies, which are consequences of the existence of genetic polymorphisms that can influence not only the propensity for certain disease conditions but can also modify the pharmacokinetic and pharmacodynamics of one drug, reducing or increasing it bioavailability (Fleischer S. *et al*, 2008). In other words, the genetic variation that exists between breeds of dogs results in a risk of breed-related differences in the effectiveness and toxicological responses to several xenobiotics (Licinio J. & Wong M., 2002).

2.2.1 Linking pharmacovigilance and pharmacogenetics

It is possible to say that these two scientific branches, pharmacogenetics and pharmacovigilance, in their essence aim to understand the "heterogeneity" and population substructure of drug efficacy and safety signals. In Humans pharmacovigilance drugs which are frequently cited in ADR studies (59%) are reportedly metabolized by at least one enzyme with a genetically polymorphic variant allele, known to be associated with altered drug metabolism (Alvarado I., Wong M. & Licinio J., 2002). So, it is obvious the existence of benefits for both scientific brands: pharmacogenetics analysis can add a more mechanistic insight on pharmacovigilance reports and contribute to causality assessments; pharmacovigilance system collects data that may give clues about the possible existence of genetic polymorphisms between some breeds. Thus, the common aim is recognizing genetic factors associated with ADRs with all drugs, and effectively incorporate pharmacogenetics into the practice of pharmacovigilance (Alfirevic A. & Pirmohamed M., 2007).

According to current definitions described above, the objectives of pharmacovigilance are perfectly consistent with pharmacogenetics definition because they allow the detection and prediction of ADRs and are a tool that can reduce the risks associated with ADRs.

2.2.2 Genetic polymorphisms

Genetic polymorphism is defined when there are two or more allelic forms in the same population and the commonest allele locus is polymorphic with a frequency of 0.99 or less. Defined in these terms, this implies that at least 2% of the Human population will be

heterozygous at a polymorphic locus (Hilfiker R., 2006). The main forms of polymorphism consists in: single base polymorphism (SNP - single nucleotide polymorphism), variation in copy numbers of DNA segments (CNV - copy number variation), insertion and deletion variation (INDEL) and variable number of tandem repeats (VNTR - variable number of tandem repeats). The SNPs are the most common form of polymorphism and the mutation takes place in a single pair of nucleotide bases of the DNA molecule which change a single amino acid. This is sufficient to modify, for example, the expression of an enzyme (catalytic activity and stability) and / or specificity of the enzyme substrates (Martignoni M., Groothuis G. & Kanter R., 2006; Harris H., Hopkinson D. & Luffman J., 2006; Chaves A., 2011).

The field of pharmacogenetics began with a focus on drug metabolizing enzymes, but it has been extended to membrane transporters that influence drug absorption, distribution, and excretion (Kerb R., 2006). Taking the drug metabolizing enzymes as an example, the different mutations that occurs give rise to different phenotypes between populations, for example, different individuals can have diverse levels of activity of some enzymes. Several individuals may be poor metabolizers, when there is lack of the active enzyme (in quality or quantity), usually associated with autosomal recessive characteristics; others can be extensive metabolizers, associated with autosomal dominant traits and whose enzyme activity is normal or increased; finally it can exist the ultra-rapid-metabolizers, which have multiple copies of the gene encoding a particular enzyme (Harris H. *et al*, 2006; Chaves A., 2011). Some examples of genetic polymorphisms within breeds of dogs, which can result in different responses to drugs, are presented on table 2.

Relatedly to membrane transporters, Pgp is the best-characterized ABC transporter (MDR1, ABCB1). However, new polyspecific drug transporters are being investigated and have the potential for overlapping substrate specificities and for tissue-selective expression. Developments in high throughput DNA sequencing technologies gave an affluence of information on the occurrence and frequency of drug transporter polymorphisms. Perhaps, the greatest impact of genetic transporter variants is in determining the individual susceptibility for toxic injury under some extreme circumstances, for example, during intoxication cases or during chemotherapy treatments (Kerb R., 2006).

Pharmacogenetics research of human drug-metabolizing enzymes has afforded valuable insights into the clinical pharmacokinetics of a wide range of drugs and led to a clearer understanding of the metabolic basis of drug interactions and individual susceptibility to drug toxicity and efficacy. The same impact is likely expected, relatively to the clinical implications of genetic variations in drug transporters.

Table 2: Some examples of breed related metabolic differences (adapted from: Fleischer S. et al, 2008).

Breed	Enzyme or Gene Linked	Genetic Finding/Metabolic Effects
Beagle	CYP1A	Genetic polymorphisms on CYP1A2 result in a variance of this enzyme expression. Poor metabolizers and extensive metabolizers result in significant polymorphic hydroxylation of a novel benzodiazepine.
Greyhound	CYP2B11	Deficient on CYP2B11. Canine CYP2B11 is responsible for propofol hydroxylation in dogs.
Labrador Retriever	TPMT	Statistically considerably higher RBC thiopurine Retriever S-methyltransferase activity than other breeds. TPMT detoxifies azathioprine metabolites. Low TPMT activity puts animals at risk for toxicity when using thiopurines. Nine unequivocal haplotypes are identified in dogs.
Bedlington Terrier	Murr-1	Affected dogs have chronic hepatitis resulting from a primary defect in copper excretion and abnormal copper retention in the hepatocytes. May affect drugs metabolized by the liver.
Mixed breeds	CYP2B11	14-fold variance on CYP2B11 activity in mixed breed dogs. Mixed breed dogs tended to span range of Vmax seen in Beagles (high) and Greyhounds (low).

Abbreviations: CYP: Cytochrome P450 oxidative enzymes. For example, the nomenclature, CYP2D6 refers to the enzyme (CYP), gene family (e.g., 2) subfamily (D), and individual gene (6); Vmax: Maximum velocity of an enzymatic reaction, RBC: Red blood cell; TPMT: Thiopurine methyltransferase.

2.2.2.1 Multidrug resistant: the best-characterized ABC transporter (MDR1)

At the beginning, multidrug resistant cells were defined as having drug accumulation deficit and the responsibility was attributed to a membrane glycoprotein. At first the deficit was thought to be due to a fault in permeation and the glycoprotein was named P because "permeability". The gene for this, MDR, has been cloned and sequenced, the amino acid has been sequenced and amino acid sequence has been derived (Ferry D. & Kerr D., 1994; Sakaeda T., 2005).

The identification of MDR1 gene showed the presence of polymorphisms. The different forms codify a diversity of Pgps, designated isoforms and in the Human species there are two types: MDR1 or ABCB1 and the MDR3 or ABCB4 genes (Chin J., Soffir R., Noonkan K., Choi & K. Roninson I., 1989; Mealey K, 2006). However, a large sequence homology between Pgp codified by different MDR genes is noticed (more than 70%), and they can be classified in two or three classes, according to the author (Georges E., Bradley G. Gariepy J. & Ling V., 1990; Musset S., 2002).

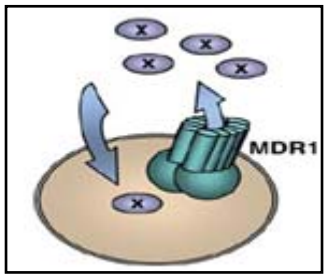
Table 3: Multigenetic MDR family (adapted from: Musset S., 2002).

Specie/ Class	I	II	III
Humans	MDR1	Ø	MDR2/3
Hamsters	Pgp1	Pgp2	Pgp3
Mice	mMdr1a (mdr3)	mMdr1b (mdr1)	mMdr2
Rat	rMdr1a (Pgp1)	rMdr1b (Pgp2)	rMdr2

Multidrug resistance transporters belong to the evolutionarily conserved family of the ATP binding cassette (ABC) proteins, present in almost all living organisms from prokaryotes to mammals. MDR transporters are large, membrane-bound proteins, built from a combination of characteristic domains, including membrane-spanning regions and cytoplasmic ATP-binding domains. On the basis of a great deal of clinical and experimental work, it has been established that these pumps recognize a very wide range of drug substrates. Although recognized substrates are mostly hydrophobic compounds, but MDR pumps are also capable of extruding a variety of amphipathic anions and cations.

These proteins play indeed an important role in cancer drug resistance. However, the biology system did not develop these multidrug transporters to protect cancer cells from medical interventions, of course, they are only co-opted and "misused" by the rapidly dividing cancer cells, especially when a population of malignant cells over-expressing an MDR transporter selected by drug treatment. With regard to their physiological role, MDR transporters most probably evolved as complex cellular defense systems, for the recognition and the energy-dependent removal of toxic agents entering the living cells or organisms from their environment (Sarkadi B., Homolya L., Szakács G. & Váradi A., 2006).

Figure 4: Multidrug/ ABC transporters (from: Staud F., Ceckova M., Stanislav M. & Pavek P., 2010).



Description: Multidrug/ ABC transporters reside in the plasma membrane and extrude various hydrophobic and/or amphipathic xenobiotics and metabolic products. MDR1/Pgp transports hydrophobic compounds (X).

The MDR1 or ABCB1 gene is located at chromosome 7q21 and this gene is not only expressed in cancer cells but also in normal tissues with excretory function and it is assumed that it has a protective function against xenobiotics (Staud F. *et al*., 2010). For example, one study shows that intestinal Pgp in humans limit the absorption of the immunosuppressant cyclosporine, cardiac glycoside digoxin and talinolol (Pang K., 2003).

Subsequently in this thesis the long known sensitivity of Collies and related breeds to the central nervous system (CNS) depressant side effect of ivermectin will be taken as an example. Previous studies had shown no important differences in pharmacokinetics of ivermectin between sensitive and non-sensitive Collies, suggesting a role for either enhanced CNS sensitivity or enhanced brain penetration of drug in susceptible animals or even both (Geyer J. *et al*, 2005). In these cases, despite the widely use of avermectins in veterinary medicine, veterinarians have followed the adage “white feet, don’t treat.” when refers to the known sensitivity of Collies (both rough and smooth). The adage has also been applied to many other herding breeds and has prevented veterinarians from using these drugs in situations where they would have been ideal (Dowling, P., 2006).

2.2.3 Discovery of MDR1 in canine specie

Rapidly, the scientists tried to find a homologue of Human Pgp in dogs, whose purpose was to use the canine specie as a scientific model to human.

In 1989, a monoclonal antibody C219 which recognizes Pgp in plasma membranes of multidrug-resistant Chinese hamster ovary cell lines was used to assay renal brush border membrane for the presence of cross-reactive polypeptide (Lieberman *et al*., 1989). The corresponding human kidney brush border membrane and dogs kidney brush border membrane proteins had molecular weights, respectively, of 170 kDa and 160 kDa. The molecular weight, antibody cross-reactivity, glycosidase sensitivity and lectin binding, demonstrated that this protein is a normal kidney analogue of the Pgp induced in multidrug

resistant cell lines in Humans (Lieberman *et al*., 1989). Later, in another study intended to characterize the canine MDR1 mRNA Human homologue, concluded that canine transcript was 4.5 Kb with 93% sequence homology to human MDR1, and 90% homology to mouse and hamster equivalent genes (Steingold S. *et al*., 1998). More, starting by documenting the MDR1 cDNA wild-type canine sequence the scientists concluded that Human and dog proteins display 91% overall homology, with nonconsensus residues being located outside the functional segments (Roulet A. *et al*., 2003; Bourassi G., 2008).Then, the MDR1/Pgp exists in canine specie and it is similar to Humans.

2.2.3.1 MDR1 gene deletion in mice

Given the importance of MDR in tumor cells resistance to chemotherapy, through the Pgp, several studies have tried to find a way to inhibit this gene (Kankesan J. *et al*., 2004; Kankesan J. *et al*., 2003). One article describes the experimental work where the MDR1a gene in mice was disrupted and its consequences were observed. The knockout mice were viable and fertile and appeared phenotypically normal, however, they displayed an increased sensitivity to the centrally neurotoxic pesticide ivermectin (100-fold) and to the carcinostatic drug vinblastine (3-fold). It was also observed that in mutated mice there were high drug levels in many tissues, especially noticed in the brain (Schinkel A. *et al*., 1994).

2.2.3.2 Discovery of MDR1 mutation in Collies

Subsequently to the results obtained above, a quick parallelism was made with the Collie breed and their extreme sensitivity to ivermectin and other molecules.

During a famous and unplanned experiment, in Netherlands Cancer Institute's animal department, the animal cages were sprayed with ivermectin routinely used to treat mite infestation. Ivermectin was supposed to kill only nematodes and arthropods, but the treatment left all the knockout mice, for MDR1, dead. The scientists reproduced the experiment and verified a 50- to 100 fold more sensitivity of MDR1a homozygous mutated mice, due to an increased accumulation of the drug in the brain. Afterwards, two independent groups searched and found this 4-bp deletion (AGAT) in the MDR1 gene on ivermectin-sensitive Collies.

According to Roulet *et al.* (2003) this nt230 (del4) MDR1 involves a frame shift at amino acid position 75, followed by a premature stop codon at 91 amino acid position. This severely truncated protein is nonfunctional, so Adenosine triphosphate (ATP) hydrolysis, which provides the energy for active drug transport, can function against steep concentration gradients. Furthermore, it was demonstrated in 17 Collies that all dogs with a homozygous MDR1 mutation showed the ivermectin-sensitive phenotype but none of the Collies with a

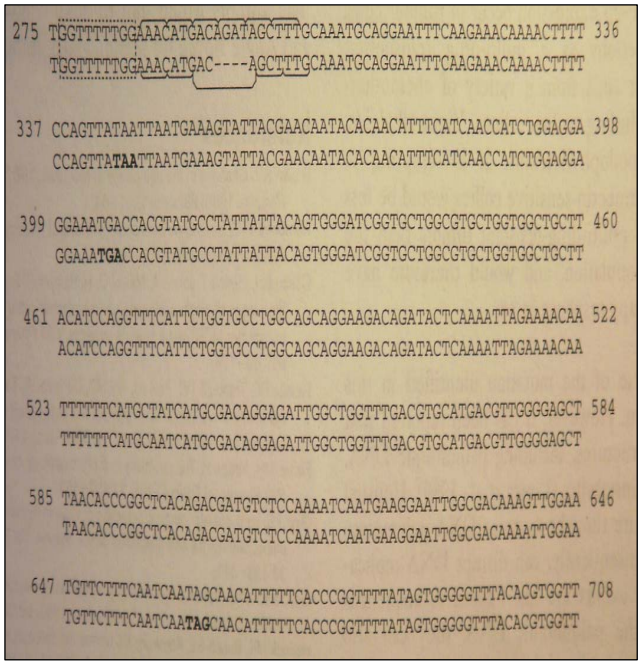
heterozygous MDR1 mutation or the wild-type genotype, pointing to an autosomal recessive inheritance pattern (Mealey K., Bentjen S., Gay J. & Cantor G., 2001).

Similar to Humans, the canine specie has two alleles for each trait. These alleles can be dominant or recessive and there are three possibilities for the MDR1 canine gene: they can be homozygous recessive (mutant/mutant), heterozygous (normal/mutant), or wild-types (normal/normal). Dogs which are homozygous recessive for the MDR1 mutation have nonfunctioning Pgp and, therefore, they have a slight but crucial altered pharmacokinetic and toxicity profiles for Pgp substrates. This glycoprotein can also impact canine medicine in ways unrelated to the MDR1 mutation, as for example, the failure of prednisolone to successfully treat naturally occurring chronic canine enteropathies, in various dog breeds, that could be predicted by the over-expression of Pgp in the dog's *lamina propria* lymphocytes during steroid exposure (Mealey et al.,2001).

2.2.3.3 Causes for the presence of MDR1 mutation allele within the canine specie

The cause for this mutation is unknown, however, it has been reported that unusual DNA structures, named palindrome, are the main reason for the existence of genetics instabilities. Mealey *et al*. (2001) noted that a palindrome is located in 9 bp upstream of the MDR1 gene mutation and that sequences sometimes provide mutational hot spots. Additionally, in this study was also verified that all Collies have the same mutation. Given that and the fact that they were not close relatives it was concluded that mutated alleles have a common origin, which will take place within the palindrome, but also close to it. To test allelic associations, MDR1 was mapped by radiation hybrid analysis and the four closest markers were selected to genotype individuals from affected breeds. Only one type of MDR1 mutation has emerged in this canine breeds (Geyer J. et al, 2000).

Figure 5: Comparison of two genetic sequences: with the mutation and without it, (from: Mealey *et al* 2001).



Description: Sequence (bases 275- 708) comparison of wild-type (top) and mutant (bottom) MDR1 canine DNAs. A 4-bp deletion is present in the mutant canine DNA. Codons in the vicinity of the deletion are indicated by brackets for both the wild-type and mutant canine DNAs. Bold letters shows stop codons created in the mutant canine DNA as a result of the frame shift. The dashed box shows the palindromic sequence in the immediate area of deletion mutation.

The sensitivity to ivermectin and other drugs was finally linked to a single mutation on MDR1 gene, that encodes a particularly glycoprotein which has a central role. Its absence or its modification leads to an accumulation of the molecule in the CNS and to the neurotoxic effects that will be detailed later. Following, this glycoprotein will be described in more detail.

2.2.4 The important role of P-glycoprotein in pharmacogenetics

The ATP-binding cassette transporters belong to a super-family composed of more than 100 membrane transporters/channels and the members of this family play a central role in cellular physiology. They are highly expressed in numerous organisms, from bacteria and plants to mammals. In humans, 48 ABC transporters have been identified, and classified on the basis of phylogenetic analysis into 7 subfamilies, and Pgp is a member of the ABCB subfamily (Ambudkar S., Kim I. & Sauna Z., 2006; Hennessy M. & Spiers J., 2007).

As mentioned above, there are different Pgp isoforms, which have more than 70% sequence homology, encoded by a small family of closely related genes. In humans, Pgp is encoded by two multidrug resistance genes, MDR1/ABCB1 and MDR3/ABCB4, but the multidrug-resistant phenotype is associated only with the first (Gottesman M., Pastan I. & Ambudkar S., 1996; Hennessy M. & Spiers J., 2007). In dogs only one gene has been identified, the MDR1 (Musset S., 2002).

One study produced by Bendayan *et al*. (2006) due to the high sensitivity of immunocytochemical, allowed the knowledge about Pgp biosynthesis. This synthesis takes place within rough endoplasmic reticulum, as a core glycosylated intermediate with a molecular weight of 150 kDa. Subsequently, within the Golgi complex, glycosylation and phosphorylation occur and their roles are not well identified. Probably, these phenomena are associated with Pgp stabilization, function and capacity to export to the cell surface (Ambudkar *et al.,* 2006; Bendayan R., Ronaldson P., Gingras D. & Bendayan M., 2006).

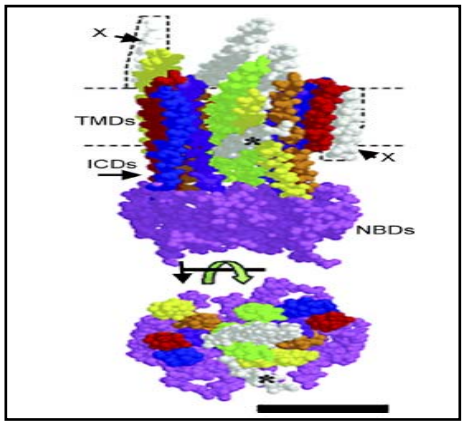
2.2.4.1 P-glycoprotein structure and ATPase fraction

Studies were mainly done in the Human species but the high homology of Pgps between Humans and dogs permits an extrapolation. Human and canine Pgps are very similar and the difference between them relies in the glycosylation process. Consisting of 1280 amino acids organised in two tandem repeats of 610 amino acids and joined by a linker region of approximately 60 amino acids (Gottesman M. *et al*, 2006; Johnstone R., Ruefli A. & Smyth M., 2000), the protein has arisen by a gene duplication event, fusing two related half molecules, each consisting of one nucleotide-binding domain and one transmembrane domain (Hennessy M. & Spiers J., 2007).

The secondary and tertiary structures of Pgp have not been fully elucidated. Many models have been suggested, some of them contradictory. All these models are suspected to exist in different proportions in the cells, or that certain forms exist only in certain tissues. Another possibility is that some studied models were not representative of what happens *in vivo*.

However, recently the first three-dimensional propose structure of Pgp was constructed. At 0,8 nm resolution, Pgp is comprised of two transmembrane domains made up of six α-helices and, five of the six α-helices from each transmembrane domain, displays a pseudo-two-fold symmetry, while the sixth breaks symmetry. It also suggests the presence of two globular areas at the cytoplasmic side of the protein, representing the nucleotide-binding domains, figure 6 (Henessey, 2007; Rosenberg M., Callaghan R., Modok S., Higgins C., & Ford R., 2005).

Figure 6: Space-filling model of Pgp in the nucleotide-bound form based on cryoelectron microscopy data and with all residues modelled as alanine (from: Rosenberg M. et al., 2005).



Description: Lateral view of the protein with the nucleotide-binding domains (violet) at the bottom. Below, a top view. The 12 putative membrane-spanning α-helices have been colored in pairs to specify the 2 halves of the transporter. A pseudo-symmetry relationship is seen. The gray-colored helices do not show an obvious symmetry relationship. Scale bar = 5 nm.

Drugs interact with Pgp in different targets according to their chemical classes. The results and analyses of site-directed mutants of Pgp that influence, substrate binding or specificity, support the view that drug-substrate binding occurs mostly in the trans-membrane domains (Ambudkar S. et al., 2006). Multiple experiments showed structures with a single large cavity comprising a "drug-binding pocket" where individual "drug-binding sites" are generated by each ligand using a different subset of residues for drug-binding. The high affinity binding site or the narrow region of the funnel appears large enough to accommodate two different drugs at the same time. In addition, while these studies provide an understanding of how the drug-binding pocket may be occupied, it is not understood how the transport of these drugs occurs under conditions where more than one drug or modulator is present (Ambudkar S. et al., 2006).

Pgp is a curious translocating ATPase, the reason is because its purified protein exhibits a high level of basal ATPase activity in the absence of substrate. The substrate-inducer of ATPase activity is frequently biphasic, with stimulation at low drug concentrations, and inhibition at higher concentrations (Hiroshi O., Marwan K. & Al-ShawiHilfiker R., 2006). Recent experiments have demonstrated that 2 to 3 molecules of ATP are hydrolyzed per molecule of substrate transported and these results are close to the others values on energy-dependent-transport (Ruetz S. & Gros P., 1994).

Several models have been advanced to understand the mechanism by which Pgp and other ABC transporters couple the hydrolysis of ATP to move drugs across the plasma membrane. Resulting of experimental data, we have: the "hydrophobic vacuum cleaner", the "flippase" and the "pore" models (Ambudkar S. et al., 2006; Hennessy M. & Spiers J.P., 2007). According to "hydrophobic vacuum cleaner" theory, Pgp is envisaged as an extracting hydrophobic compounds embedded in the inner leaf of the plasma membrane and pumping

them straight to the external aqueous medium (Ambudkar S. et al., 2006). Alternatively, in the "flippase" model, the substrates are flipped from the inner leaflet of the lipid bilayer, to either the outer leaflet of the plasma membrane or straightly to the extracellular environment to the extracellular environment. In the "pore model", which has less supporters, the Pgp functions as a simple canal (Higginsa C., Gottesmanb M., 1992).

Therefore, it is assumed in both models that substrates partition into the lipid phase is prior to interacting with Pgp. This possible clarify the curiously broad substrate specificity of Pgp, since the primary determinant of specificity would be the capacity of a substrate to intercalate into the lipid bilayer appropriately, with subsequent interaction with the substrate-binding site being of secondary importance.

2.2.4.2 P-glycoprotein tissue distribution and possible roles

Pgp can be detected in human tissues by hybridization techniques, immunoblotting or immunohistochemical methods (Cunningham F., 2010).

Ginn P. in 1996 had some pertinent objectives in his study: determining if it was possible or not to detect Pgp expression with immunohistochemical methods in canine tissues, routinely processed for histopathology. Also, they determine the tissue distribution and intracellular location of Pgp expression in normal canine tissues and compare these findings with those reported in the literature for Pgp in human tissues. Monoclonal mouse-antibodies C494 (specific for MDR1), C2 19 and JSB1 (recognize both MDR1 and MDR3 according to the distributor) were used (Conrad S. *et al*., 2001). Consensus recommendations for Pgp recognition have been established and suggest as useful the use of multiple antibodies with at least two different epitopes recognition, one extracellular and another intracellular (Beck W. *et al*., 1996). The use of this method allowed the comparison of Pgp distribution in human and dog tissues, which are very similar (Conrad S. *et al*., 2001).

Table 4: Comparison of the tissue expression pattern of P-glycoprotein in human and dog, (adapted from: Conrad S. et al., 2001; Sugawara *et al*. 1997).

Tissue	Dog Pgp[a]	Human Pgp[a]
Liver	++	+
Kidney	++	++
Small intestine	**ND**	+
Duodenum	**(+)**	**ND**
Jejunum	+	**ND**
Ileum	**(+)**	**ND**
Large intestine	**ND**	+
Colon	+	**ND**
Lung	+	+
Brain	++	+
Testis	+	+

a Data in the references were adjusted as follows: ++, high expression; +, medium expression; (+), weak expression/weak immunoreactivity; ND, not determined.

Quantification of Pgp expression by immunohistochemistry is problematic, mostly in small clinical samples with low Pgp expression. However, it has been shown that reverse transcription polymerase chain reaction (RT-PCR) is appropriate for quantification of MDR1 gene expression in various human tissues, even in small clinical samples with low MDR1 expression. Currently, the establishment of quantitative RT-PCR has been very laborious. Though, the introduction of fluorescence-based detection of real-time RT-PCR products has simplified the process of generating accurate, reproducible and highly sensitive quantitative data on mRNA expression levels. A real-time RT-quantitative PCR method was established, with accurately and reproducibly, to quantify the expression level of MDR1 in normal and neoplastic canine tissues (Culmsee K., Gruber A., Samson-Himmelstjerna G. & Nolte I., 2004).

Pgp is located primarily in the intestine, liver, kidney and brain and the tissue distribution of a protein can yield important clues to understand its function. Pgp is found in polarized epithelial cell layers, where they normally localize to the apical or luminal membrane domain of the cell. This localization proposes that Pgp is mainly involved in the extrusion of some substrate from the epithelial cell layer into the adjacent luminal space (Ginn P.,1996).

Table 5: Normal canine tissues selected for Pgp immunostaining (adapted from: Ginn P., 1996).

Epithelial Tissues	Mesenchymal Tissues
Skin	Cardiac muscle
Mammary glands	Smooth muscle
Liver	Skeletal muscle
Prostate	Lymph node
Esophagus	Spleen
Stomach	Tonsil
Small/large intestine	Fibrous connective tissue
Pancreas	Blood vessels of brain
Gallbladder	
Lung	
Uterus	
Kidney	
Urinary bladder	
Testis/ovary	
Thyroid	
Adrenal	

Because of Pgp particularly distribution and function, it is possible to deduce its body protector role against xenobiotics, by limiting the entry of toxic substances in the organism (digestive tract), remove substances from the organism (kidney and biliary system), allowing the removal of substances from the tissues (placenta, blood-brain barrier) and protecting cells against substances present in interstitial space (Schinkel A. *et al*., 1994; Schinkel A., 1997).

Given the protective character of Pgp, in human patients carriers of the MDR1m, have been described some susceptibilities to diseases such as Parkinson's disease, inflammatory bowel disease, refractory seizures, and others (Mealey K., 2004).In homozygous knockout mice for the MDR gene a particular sensitivity to the development of inflammatory colitis, resembling those seen in Crohn's disease in humans, is noticed in these animals. These findings conducted to the hypothesis of the same predispositions in dogs that are affected by the identical mutation (Karen, L., 2008). More, patient with MDR1 homozygous mutation brings clinical implications to the veterinary medicine, because normal clinical doses can be toxic for them (Mealey K. et al., 2006). So, its absence/modification increases the tissues exposure to several toxic xenobiotics, with their consequences, as it has already been mentioned in this thesis.

2.2.4.3 Multidrug resistance to anticancer treatments

Several studies continue to investigate other different roles of Pgp, such as the cells resistance to virus, like HIV and others retrovirus (Raviv Y., Puri A. & Blumenthal R., 2000). However, the cells capacity to resist to anticancer treatments is the main problem in Human medicine.

A number of studies showed the presence, normal or overexpressed, Pgp in tumor canine cells samples. These cells are able to use, in their advantage, the transporter detoxification role, by producing a large number of Pgps, avoiding the anti-cancer drugs, which are Pgp substrates.

Table 6: Canine neoplasms selected for immunostaining (adapted from: Ginn P.E., 1996).

Epithelial tumors	Mesenchymal Tumors
Mammary gland adenomas/carcinomas	Malignant lymphoma
Squamous cell carcinoma	Cutaneous plasma cell tumor
Basal cell tumor	Fibroma/fibrosarcoma
Apocrine gland adenoma/carcinoma	Hemangiopericytoma
Hepatoma	Leiomyoma/leiomyosarcoma
Cholangiocarcinoma	Histiocytoma
Colorectal adenoma/carcinoma	Malignant melanoma
Transitional cell carcinoma	
Adrenal gland adenoma	
Thyroid gland adenocarcinoma	

Previous studies in Humans, using clinical material, have shown high levels of expression of MDR mRNA in tumours derived from cells normally possessing Pgp. Immunopathological studies were done on an extensive variety of tumors and Pgp expression is mostly found in tumors derived from tissues normally expressing this protein, such as carcinoma (renal, bladder, colon, gastric, hepatic, lung, adrenal, embryonal and terato), seminoma and sarcoma. Curiously, despite the consistently intense expression of Pgp in placental trophoblasts, the three gestational trophoblastic tumors examined had no detectable Pgp. The intensity of Pgp expression can diverge from one sample to another for a given tumor type, and considerable heterogeneity of expression is usually seen within a given tumor. Such a pattern of Pgp

expression in tumor cells is consistent with the phenotypic variability commonly observed in human neoplasms. Different studies point for one promising unexplored therapeutic approach in cancer treatments.

In view of these findings, it would be interesting to know the consequences of MDR1 gene inactivation in humans using animal models such as mice or dogs. As referred above, some dog breeds have this mutation in their population, which is similar to gene invalidation. So, these breeds are particularly interesting to model the inactivation effect. Other strategy, however, is selectively inactivating the MDR1 gene in tumor cells.

2.2.4.4 Substrates and inhibitors of P-glycoprotein

Mammalian Pgp has extensive substrate specificity, transporting a number of drugs with diverse chemical structures, including anticancer agents, immunosuppressants antiparasitic agents, steroid hormones and others. The mechanism behind the process of recognition and transport of diversity compounds by Pgp is still unknown (Hrycyna C., 2001). It has been a challenge to predict whether a drug will be a Pgp substrate or not based purely on chemical structure. Interestingly, the majority of this subtracts are natural compounds or synthetic derivatives of natural compounds (Wang Z., et al., 2011).

Linking numerous studies it is possible to construct a table containing a list of drugs relevant to veterinary medicine that are Pgp substrates. More, Pgp substrate specificity appears to be relevant across species, for example, drugs determined to be substrates for murine Pgp are also substrates for both human and canine.

Table 7: Summary of Pgp substrates (adapted from: Mealey K. et al., 2006; Sakaeda T., Nakamura T. & Okumura K., 2002).

Anticancer agents • Actinomycin D • Etoposide • Docetaxel • Doxorrubicin • Daunorubicin • Irinotecan • Mitomycin C • Mitoxantrone • Paclitaxel • Teniposide • Vincristine • Vinblastine • Vindesine • Topotecan	**Opioids** • **Loperamide** • Morphine • Butorphanol **Immunosuppressants** • Cyclosporine A • Tacrolimus (FK506) • Rapamycin **Anti-histamine (H1 and H2)** • Terfenadine • Cimetidine • Ranitidine **Anti-emetics** • Domperidone • Ondasetron
Steroid hormones • Aldosterone • Hydrocortisone • Cortisol • Corticosterone • Dexamethasone • Methylprednisolone	**Cardiac drugs** • Digoxin • Digitoxine • Quinidine • Adrenergic β-antagonist • Bunitrolol • Talinolol
Cholesterol-lowering agents • Atorvastatin • Lovastatin **Calcium channel blockers** • Diltiazem • Verapamil and derivates	**HIV protease inhibitors** • Amprenavir • Indinavir • Nelfinavir • Ritonavir • Saquinavir
Antimicrobial agents • Erythromycin • Ketoconazole • Itraconazole • Tetracycline • Doxycycline	**Miscellaneous** • **Ivermectin and other avermectins** • Acepromazine • Vecuronium • Colchicine • **Emodepside**

Within these molecules referred on table 7, will be listed in more detail the loperamide and the emodepside, because they were chosen for analysis in the practical part of this dissertation.

Loperamide is a weak opioid with low analgesic activity. It has licensed use in veterinary medicine as a treatment for non-specific chronic and acute diarrhea. It also has human use as an antidiarrheal drug and as an adjunct to rehydration therapies (Campbell A. & Chapman M., 2000). Several cases concerning poisoning with loperamide in Collies and other breeds have been documented and they presented an identical symptomatology with the ivermectin cases, affecting mainly the nervous system and similar symptomatology. In dogs, the most common side effects of opiates are constipation, sedation and bloating. Neurological disturbances such as ataxia, hyperexcitability, circling, head pressing, vocalization and prostration may occur with overdosage (Hugnet C. *et al*., 1996). There are cases where the intoxication symptoms started at lower doses than those determined for Beagles, so more than 0.63 mg / kg results in vomiting and more than 5 mg / kg results in hind-limb paresis. However, it seems that a single dose of 0.42 mg/kg, in Collies, results in profound sedation and unresponsiveness, bradycardia, respiratory depression, constricted pupils and hypothermia (Campbell A. & Chapman M., 2000). So, at therapeutic doses it has no CNS effects and does not cross BBB, which may wonder in the same mechanism as ivermectin

Emodepside is a novel substance and current knowledge about its mode of action suggests a quite different mechanism from the common anthelmintics used. Emodepside stimulates presynaptic secretin receptors resulting in paralysis and death of the parasite. In literature a possible interaction with Pgp substrates/inhibitors is described and the margin of security in Collies and related breeds is suggested to be lower than in other breeds (Ramsey I., 2010).

Among the substrates of Pgp, some molecules appear to have only a modulator, agonist or inhibitor role and it is important to know which ones have such a function and in which direction. An inhibitor is defined as a substance which is able to inhibit the efflux of other compounds or decrease the ATPase activity of Pgp. However, a lot of other complex mechanisms involving this regulation are present, and these antagonists are very useful to improve the cancer treatments or even to upgrade some medicaments witch acts at CNS level, (Mealey K., 2006). Below, a table with some important inhibitors is presented.

Table 8: Selective inhibitors of Pgp (adapted from: Mealey K., 2004; Mealey K. et al., 2006).

Antidepressants	**Cardiac drugs**
Fluoxetine	Verapamil
St. John's Wort	Amiodarone
Paroxetine	Carvedilol
	Quinidine
	Nicardipine
Opioids	**Immunosuppressive**
Methadone	Cyclosporine
Pentazocine	Tacrolimus
Antibiotics	**Others**
Erythromycin	Bromocriptine
Itraconazole	Chlorpromazine Tamoxifen
Ketoconazole	Grapefruit juice

2.2.5 Ivermectin and Collies sensitivity to this drug

Discovered in 1979 and marketed since 1981, ivermectin (an endectocide drug) revolutionized the market for parasiticides through its broad spectrum of activity and efficacy. Rapidly, its use has exceeded the frame of market authorization and the off-label use lead to several ADRs in canine species (Campbell W. & Benz G., 1984).

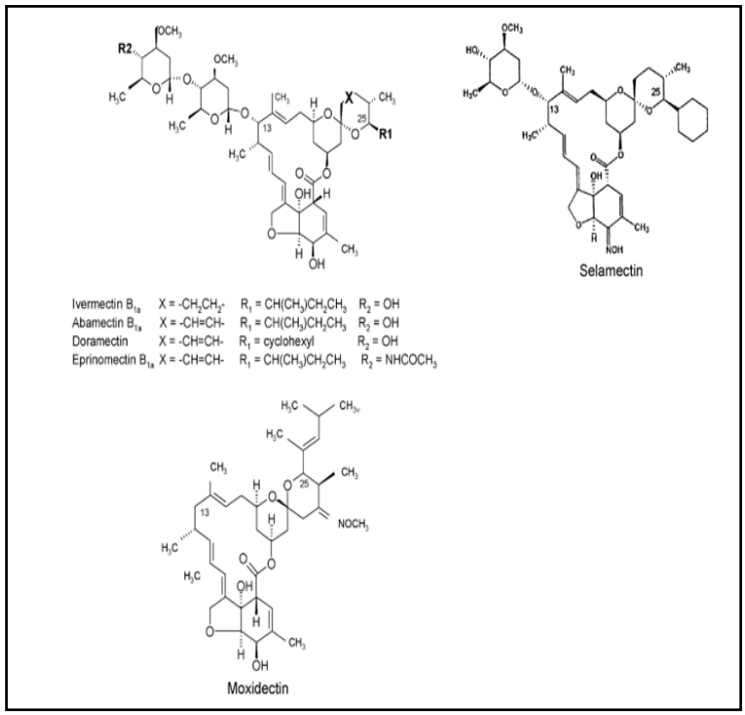
2.2.5.1 Family of avermectins

Following the research started in 1975 by Merck-Sharp-Dhome, several vitro assays for detecting fermentation products with anthelmintic activity had been run without success, mainly because of the large number of toxic compounds which had to be eliminated (Campbell W. & Benz G., 1984). In 1979, concentrates of fermentation products were administrated to be tested in *Nematospiroides dubius* infected mice and the success had come with these samples collected fom Kawana, by the Kitasato Institute. From extracts of culture, the active substance responsible for the antiparasitic activity was isolated. The name of avermectin was chosen because of its vermicide and ectoparasiticides activities and the microorganism was an unknown species of *Streptomyces,* which was named *Streptomyces avermitilis* (Burg R. *et al*, 1979). Nowadays, the industrial production uses a mutant strain of this microorganism and particularly fermentation procedures leading to the fabrication of a set of avermectins identical to those found in the original culture medium (Shoop W., Mrozik H. & Fisher M., 1995).

2.2.5.2 Structure

Avermectins and milbemycins belong to the same family of Macrocyclic lactones and share the same structure with 16-membered macrocyclic ring, which gave rise to the class name. Milbemycins were already described in 1973 with its activity against important agriculturally mites. However, their activity against nematodes was only investigated later, after publication of anthelmintic properties of avermectin (Shoop W. et al, 1995). The avermectins are disaccharides (ivermectin, doramectin) or monosaccharides (selamectin), while the milbemycins (milbemycin oxime and moxidectin) have no sugar components (Birchard S. & Sherding R., 2008). Avermectins are a mixture of 4 major components (avermectin A1a , A2a, B1a e B2a) and 4 minor components recovered in smaller quantities (avermectin A1b, A2b,B1b and B2b).Of these, the B1a component is recovered in greatest quantity along its minor homolog, B1b (Shoop W. *et al*, 1995).

Figure 7: Structures of macrocyclic lactones (from: Edwards G., 2003).



Description: For ivermectin, the dry compound is mixture of 22,23 dihydro-avermectin B1a (substituent isobutyl on C25) and B1b (substituent isopropyl on C25) forms. Abamectin, eprinomectin, doramectin are also mixture of B1a and B1b forms. The majority (over 90%) of the drug is present as the B1a form.

The structures of all the eight components contain the same disaccharide substituent at the 13-α-position. They vary at C-5 with hydroxyl or methoxy groups, at C-23 with an axial β-hydroxy group on a 22,23- olefin and at C-25 with isopropyl or sec-butyl groups in contrast to the methyl and ethyl substituents at the 25-position of the milbemycins.

With X-ray analysis of aglycone B2a and B1a, the structure was confirmed and established the absolute stereochemistry (Campbell W., Burg R., Fisher M. & Dybas R., 1984). Ivermectin derivate from the mix of B1 avermectins by saturation of the double bond between

C-22 and C-23, with more than 80% of 22,23-dihydroavermectin B1a and less than 20% of 22,2-dihydroavermectin B1b (Edwards G., 2003; Birchard S. & Sherding R., 2008).

2.2.5.3 Properties of ivermectin

Registered in France in 1981, ivermectin was the first avermectin to be introduced and possibly the most successful veterinary drug ever produced. Ivermectin, as it major component, 22,23- dihydroavermectin B1a, is a highly lipophilic and hydrophobic substance, that can dissolve in the most part of organic solvents (Plumb D., 2001).

Ivermectin is absorbed rapidly after oral administration of tablets or chewable dosage forms, with peak plasma concentrations at 4 to10 hours. Maximum concentrations increase directly with the dose indicating a linear relationship between dose and bioavailability. The drug is widely distributed, with a volume distribution of 2.4 L/kg, and is eliminated with a half-life of approximately 1.8 days. Approximately 93% of ivermectin is bound to plasma proteins. The drug is extensively converted by hepatic CYP3A4 to at least 10 metabolites, mostly hydroxylated and demethylated derivatives. The presence of ivermectin in urine (unchanged or conjugated) has not been detected and it is excreted essentially by faeces. Highest tissue concentrations occur in liver and fat. Extremely low levels are found in brain (Birchard S. & Sherding R., 2008).

2.2.5.4 Use of ivermectin in dogs

Given the efficacy, simplicity of administration and low cost of ivermectin, it was adopted by many practitioners who applied it in dogs in extra-label use. Note, that authorization market of ivermectin for dogs is only indicated when is needed to treat infection with L3 and L4 larvae of *Dirofilaria immitis* and the recommended dose is 0.006 mg / kg *per os*. Ivermectin is not active against the adult stage of the parasite. It is possible to say that at the recommended dosage, for heartworm prevention, ivermectin is safe for all dog breeds, except when acute anaphylactic reactions from microfilarial die off occur (Birchard S. & Sherding R., 2008).

This drug has also an efficacy of 96-100% against adults and larval stages of *Ancylostoma caninum* and *Uncinaria stenocephala* at dosages of 0.002 mg/kg orally. To control Ascaris, Strongyloides, and Trichuris species normal doses of 0.2 mg/kg are used. Several reports indicate that greater than 99% of *Trichuris vulpis* infections are expelled at dosages of 0.1 mg/kg and 0.2 mg/kg will remove 90% of all adult stages and 97% of the intestinal larval stages of *Toxocara canis*. To produce an effect against tissue dwelling stages of *Toxocara canis* in dogs and in mice high dosages (1-2 mg/kg) are required. Against canine ectoparasites, a single dose of ivermectin at 0.2 mg/kg produce complete cure of natural

infection of *Otodectes cynotis* and *Sarcoptes scabiei*. For treatment of sarcoptic mange, even in severe cases, two treatments at 14 day intervals have been advocated. It is also used for treat Cheyletiellosis (0, 2-0, 3 mg/Kg) and generalized demodicosis (0, 3-0,6mg/kg) (Mueller R, 2004).

2.2.5.5 Symptoms and toxic doses

Toxicological studies of ivermectin were realized to obtain the MA, and they helped highlighting about the toxic doses from which more or less severe symptoms in several species, including murine and canine, are obtained. Further information has been collected through additional scientific studies and publications of accidental intoxications. Though, in this thesis, only the acute toxicity of ivermectin will be referred.

Studies carried out on mice showed that a single administration of 25 mg/kg and 30 mg/kg, oral or parenteral, reaches the lethal dose 50 (LD_{50}). The clinical observed signs were related with the nervous system such as CNS depression, including coma and death. There were also ataxia, bradypnea and tremors (Lankas G. & Gordon L., 1983). In Beagles symptoms of acute toxicity rarely occur at single dosages of 2 mg/kg. At 2.5 mg/kg and at 5 mg/kg it occurs mydriasis and tremors, respectively. At doses of 10 mg/kg, severe tremors and ataxia are seen. Deaths occurred when dosages exceeded 40 mg/kg, but the LD_{50} is 80 mg/kg (Lankas G. & Gordon L., 1983; Plumb D., 2001).

However, as it is known, some dogs, especially collies and other herding breeds are more sensitive to ivermectin and can only tolerate doses up to 0.1 mg/kg, which is sixteen-fold higher than the label dose (Birchard S. & Sherding R., 2008).

2.2.5.6 Research of physiopathogeny: implications in CNS

As it was referred, the most important signs consequent to an ivermectin toxicosis are related with CNS. The success of this drug is linked to its mechanism of action whose origin, on arthropods and nematodes, a lethal paralysis following opening of chloride ion channels in cell membranes of peripheral nerve tissues, leading to an hyperpolarization. The glutamate-gated chloride-channels are the target site that are present in both neuronal and muscle membranes of many invertebrates, but not in mammals (Birchard S. & Sherding R., 2008). This possible model of functioning describes a first fixing step of ivermectin to its receptor (perhaps a glutamate receptor) resulting in a set of interactions with close receptors, namely the benzodiazepine and gamma amino butyric acid (GABA), allowing the release of chloride ion. As result from this interaction, paralysis of the pharyngeal pump, necessary for food intake and consequent starvation is observed in nematodes (Thisse A.J., 1995). This

preponderant GABA-mimetic action is compatible with the nervous signs observed in some intoxication in mammals.

This drug seems also to induce the release of the neurotransmitter GABA, which interferes, in a lesser extent, with neuronal transmission in parasites. It appears that GABA and its agonists interact with the cholinergic system, increasing the levels of acetylcholine in several brain areas (Kass I.,Wang C., Walrond J. & Stretton A., 1980). One study showed that after ivermectin administration in rats a significant increase in pseudocholinesterase activity exists. Intoxication clinical signs such as hypersalivation, vomiting and breathing difficulties may be due to this cholinergic function mediated GABA exacerbation (Thisse A.J., 1995).

However, at least one study suggests a depolarizing rather than hyperpolarizing role for ivermectin on the glutamate-gated chloride channel (Pemberton D. & Franks C., 2001).

Though, in either case, the end result is the deactivation of the channel by manipulation of chloride levels. Without binding sites, cestodes and trematodes are insensitive to these molecules and in mammals they are located in the CNS, but extremely low levels are found in brain (Birchard S. & Sherding R., 2008).

The presence of ivermectin and its action mode in the CNS is already known and referred in this dissertation. However, the scientists did not understand how this molecule crossed the blood-brain barrier (BBB), or it was not rejected later, turning some mammalians sensitive to this drug. Several hypotheses had been considered along time, such as, an increase in the total concentration, an increase of the free fraction or a change in the meningeal blood barrier.

2.2.5.7 Physiopathogeny in Collies

The first studies directed their research to great concentrations of ivermectin in the plasma of sensible dogs. Five Collies sensitive to toxic effects of ivermectin and 7 non-sensitive Collies were studied, and no significant differences were observed in the peak plasma concentration (Tmax) and the time to peak concentration, between the two groups. The observed toxicity in sensitive collies did not appear related to an increased absorption or a smaller elimination of the molecule (Tranquilli W., Paul A. & Seward R., 1989).

Another example has tried to demonstrate a role for plasma proteins in determining the amount of ivermectin available for transport across the BBB of collie dogs sensitive to the effects of the drug. So, the solubility of ivermectin in plasma from non-sensitive and sensitive collies was measured and the results were identical. Also, in the same experiment, an assay for measuring the low affinity binding interaction of ivermectin with plasma components was developed, and no differences in the binding characteristics of ivermectin in plasma in the two groups were found. This means that the ratio- free

ivermectin/ protein-bound ivermectin plasma concentrations- is the same between the two groups (Rohrer S. & Evans D., 1990).

Due to the main neurologic symptoms and from the studies, referred above, resulted the idea that probably the different reactions between the two groups resides on a structure or a particular physiology of the BBB in dogs considered sensitive to ivermectin and other drugs.

2.2.5.8 The blood-brain barrier

The existence of the BBB that limits exchanges between blood and brain compartments was discovered by P. Ehrlich in 1885 (Boulert C.R.G., 1992). The new medical technologies like electronic microscope and molecular tracing in the middle of XX century allowed then the precision notion of this barrier (Lepage J., 1998).

The main structures responsible for the barrier properties are the tight junctions and cells, namely, capillary endothelial cells (BBB) and the epithelial cells (glial). The BBB at the level of brain microvessels creates the largest surface area, the "barrier interface" (12–20 m^2 / 1.3 kg of brain) and has the greatest influence on drug delivery to the brain (Abbott N., 2005).

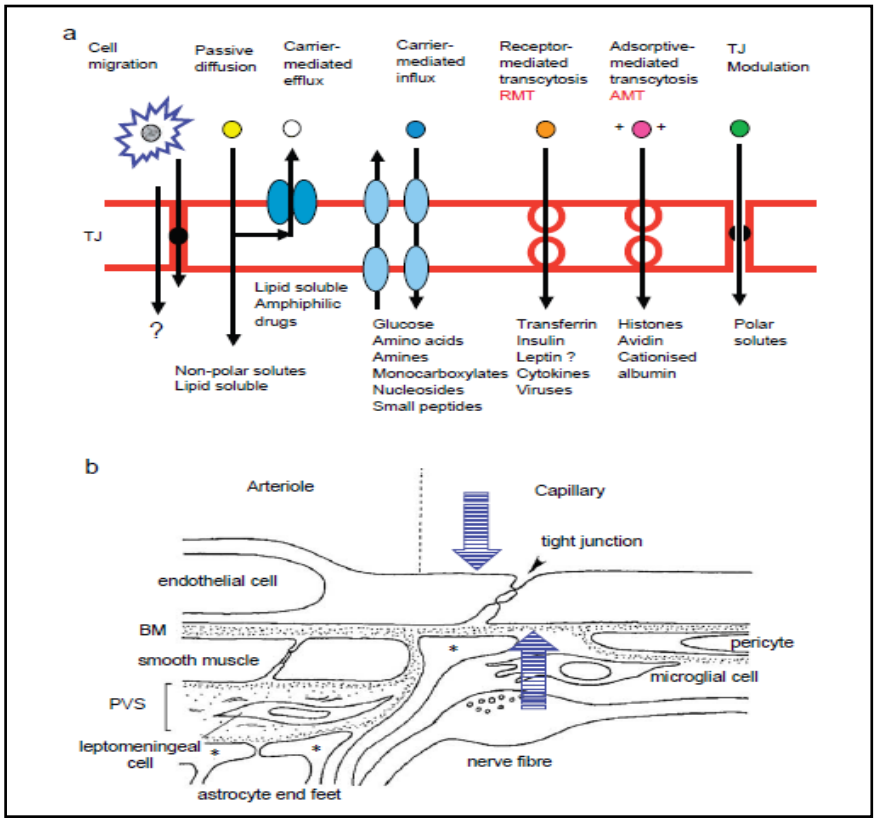
2.2.5.9 Physiology and functions of the blood-brain barrier

The BBB has different functions: ion regulation (the BBB provides a constant environment for neural function and, by a combination of specific ion channels and transporters, keeps the ionic composition optimal for synaptic signaling function), macromolecules selection (the BBB prevents entrance of many macromolecules in brain. The protein content of cerebrospinal fluid is much more low than in the plasma, and the individual protein composition distinctly different) and neurotoxic substrate brain´s protection (the BBB functions as a protective barrier which shields the CNS from neurotoxic substances circulating in the blood. These neurotoxic substrates may be endogenous metabolites, proteins, xenobiotics ingested in the diet or even acquired from the environment, which are dynamically pumped out of the brain through the ATP-binding cassette transporters) (Abbott N., Patabendige A., Dolman D., Yusof S. & Begley D., 2010). Numerous principal characteristics of the "BBB phenotype" appear to be induced in the brain endothelium via chemical influences from the associated cell types.

The flux of molecules through the BBB depends on many factors and can be done by different processes. The brain endothelium forms the BBB and is the principal site regulating molecular traffic between blood and brain. The "barrier phenotype" comprises tight junctions restricting paracellular flux and a range of transport mechanisms regulatory transcellular flux.

The barrier is induced by cell types associated with the microvessels and is subject to regulation. The permeability of lipid-soluble compounds is influenced by the composition of the BBB membranes. Specific transporter systems for solute uptake are present on both the apical (luminal) and basal (abluminal) membranes and efflux transporters of broader specificity are also present. Some larger molecules may cross via transcytotic vesicular mechanisms. Potential drug molecules designed to enter the brain may use or interact with one or more of these routes. Many lipid-soluble drugs are substrates for efflux transporters (Abbott N.J., 2005) and ivermectin is able to cross the barrier by passive diffusion, due its low molecular weight of 700 Da (Schinkel A.H., 1999).

Figure 8: Different modalities of passage of molecules and cell types present at the BBB (from: Abbott N.J., 2005).



Description: (a) Permeability and transport across the blood–brain barrier. TJ, tight junction. (b) Some of the cell types present at the BBB capable of modulating brain endothelial permeability and function; the arrows indicate that modulatory influences may come from either the blood or the brain side

The CNS barrier provides the stable fluid microenvironment that is critical for complex neural function and protects the CNS from chemical insult and damage. Thus, an open BBB can be found in some tumours and acute phases of multiple sclerosis, epilepsy (during seizures) and up-regulation and changed distribution of drug resistance efflux transporters have been also reported. Then, the question remained, what fails in the BBB that permit the passage of ivermectin or its persistence in the CNS of sensitive dogs?

One study for the first time took as hypothesis a mechanical change on the BBB, and after 200 μ/kg of ivermectin having been orally administered, two groups of Collies were formed, one with neurotoxic evidences and other without. It was measured the cerebrospinal fluid pressure and neurotransmitter metabolic concentrations in the *cisterna magna,* 49 to 50 hours after administration. No significant differences between the intracranial pressures were found, raising the hypothesis of an anatomic or physiologic modification (Gallo J.M., Li S., Guo P., Reed K. & Ma J., 2003). However, despite the several studies directed to understand this event, which involves a simple DNA mutation resulting in a non-functional glycoprotein, was achieving only with the accidental experience, described above, in the Netherlands Cancer Institute's animal department.

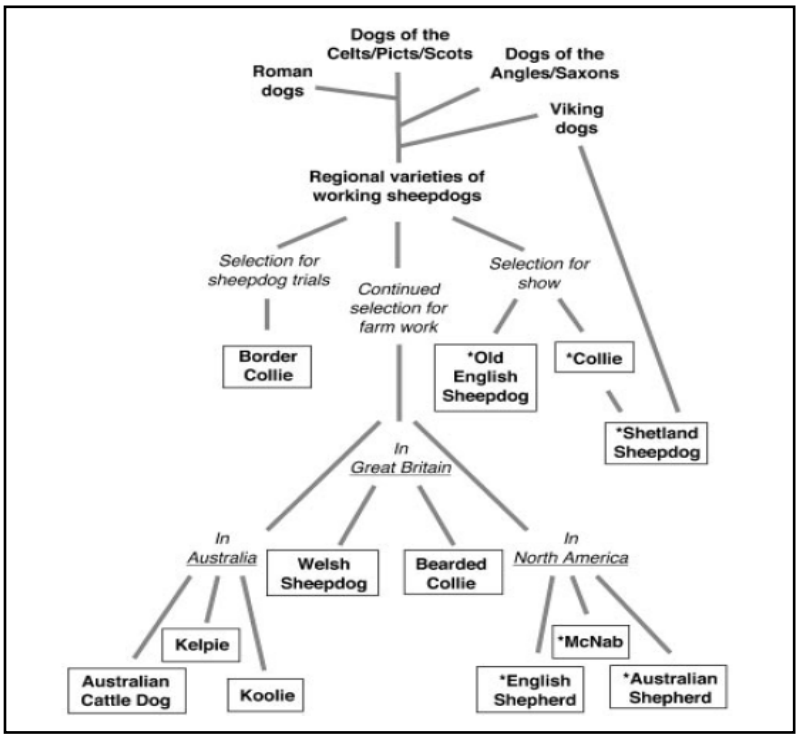
2.2.6 Phylogenetic studies

A mutation in the canine MDR1 gene has previously been associated with drug anomaly sensitivities in two breeds from the Collie lineage. Though, more breeds have also these SARs to ivermectin and other drugs, some already referred above, and the question of a common origin of the mutation arose, especially since most races are related to Collie.

2.2.6.1 Exploring the phylogeny of breeds

The exploration of breed phylogeny, report drug sensitivity and analyses of other purebred populations that are probably at genetically risk, was study previously. A survey of dog populations based on phylogeny (supposed relatedness to the Collie) and phenotype (reports of drug sensitivity) was produce. Four classes of dogs were constructed and tested: 1) breeds from the collie lineage that were selected based on a complex of breed histories; 2) European herding breeds that, supposedly are not closely related to the Collie; 3) Sighthounds and various breeds that had exhibited drug sensitivities, often in response to ivermectin and 4) a multibreed panel with more than thousand samples from 90 breeds. This group was incorporated to create a general baseline of MDR1 frequency among purebred dogs (Neff M. *et al*., 2004).

Figure 9: Collie Family Tree, a composite of subjective breed histories (From: Neff M. et al., 2004).



Description: 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.

In this study, it was found that at least nine breeds of dog segregated an identical-by-descent allele of MDR1 that predisposes dogs to multidrug sensitivity, breeds are: Australian Shepherd, Australian Shepherd Miniature, Collie (Rough and Smooth), English Shepherd, Longhaired Whippet, McNab, Old English sheepdog, Shetland Sheepdog and Silken Windhound. Approximately, half of breeds from the published Collie Family Tree, was generally predictive of the observed distribution of MDR1, thought, the mutation was not found in tree breeds whose origins traced back to continental Europe, like Border Collie, Bearded Collie and Australian Cattle Dog. But, there are records of individuals from these breeds with high sensibility to ivermectin and possible explanations are the existence of another mutation or a low percentage of mutation carriers, as shown by Geyer J. *et al* (2005) experiences, where the mutation was found in Border collie, however, not in Bearded Collie. Later, two cases were reported also by this team, describing the presence of the same mutation in the White Swiss shepherd dog, tested after their unusual sensitivity to doramectine (Geyer J. *et al*., 2007). In another study, realized in the canine Belgian population, it was searched the MDR1 mutation in diversified suspected breeds and genotyped 92 dogs. Results were similar in its frequencies to other studies realized in several countries, (Erkens T. *et al*., 2009).

Table 9: Resume of breeds which MDR1 mutation has been documented (adapted from Mealey K. *et al.,* 2006).

Herding breeds	Collie (Rough and Smooth), Australian shepherd (standard and miniature), English shepherd, McNab, Old English sheepdog, Shetland sheepdog, German shepherd, White Swiss shepherd and Border collie[a].
Others	Longhaired whippet, Silken windhound and White Swiss shepherd dog.

a Based only on dog's appearance.

2.2.6.2 MDR1m and history of a common origin

Dogs carrying MDR1m share a common ancestor that experienced an extraordinary evolutionary success, having contributed genetically to at least nine distinct breeds of dog. Possibly, this animal lived in Great Britain in the 1800s, previous to the emergence of official breeds. Before 1870, there were no established registries for sheepdogs, only regional varieties of working dogs that had been adapted to terrain, climate, breed of sheep, and working style. In the 19th century, the industrialization process brought changes in trade and transportation that may have facilitated a mixture among these varieties. The function of working dogs (drive sheep to markets) was no longer needed after de socioeconomic changes. The negligence of regional varieties may have contributed to the beginning of dog shows, which aimed to preserve and restore strains by emphasizing appearance rather than function. The first formal breeds to emerge from working sheepdog populations were the Collie, Old English sheepdog, and Shetland sheepdog. Working Collies contributed genetically to the Shetland sheepdog, which probably accounts for the presence of MDR1 in the latter breed. Therefore, the allele probably has already been prevalent among working collies by the 1890s. The Old English sheepdog was a founding member of the Kennel Club of England in 1873, and has probably been genetically isolated from other collie-related breeds since that time. Contrasting, the Shetland sheepdog, the Old English sheepdog are distinct from the Collie in size, shape, and behavior, so register show that Collies are improbable to have been the cause of MDR1m. Rather, admixture among the working progenitors of these two breeds is the more liable explanation.

In the same century Great Britain describes shepherds as using two types of dogs: the smaller collies that excelled at herding and the larger more versatile ‘‘old English type’’ that could drive, protect, and herd the flock. The use of both types by shepherds presumably afforded gene flow. Thus, the ancestral population that produced MDR1m was probably an admixed

population of working sheepdogs. The ancestors of the Australian Shepherd, English Shepherd, and McNab also trace back to this ancestral population, generally defined. Although these latter breeds were developed in North America in the 1900s, they were most likely derived from nondescript farm Collies imported from Great Britain and Australia in the 1800s and early 1900s.

The high frequency of MDR1 in both subpopulations of Collies, the broad distribution of haplotypes I and II among multiple breeds, and the distinct haplotypes of the Old English sheepdog together suggest that MDR1m was extensively dispersed by the time breeds were being registered, possible in 1873.

The attendance of the mutation in Sighthounds might provide a more recent historical perspective on MDR1. Initially, reports of ivermectin sensitivity in these dogs were explained as a result of crosses between Queen Victoria's Collies and Borzois given to her by Czar Nicholas II. However, the data point to a more recent event. The old Longhaired Whippet was apparently restored in the 1950s by a single breeder who also bred Shetland Sheepdogs. It is hypothesized that MDR1 accompanied an allele for long hair during focused introgression, through either linkage or drift. The Longhaired Whippet and Shetland sheepdog favour haplotype II, and this is consistent with the suggested theory. The Silken Windhound was developed even more recently (in the 1980s) by crossing multiple Sighthounds breeds, including the Borzoi, Whippet, and Longhaired Whippet (the probable source). This explanation is also consistent with a preference for haplotype II in both breeds (Neff M. *et al*., 2004).

The White Swiss shepherd dog has presented the same haplotype, which is consistent with one recent mutation acquired. This haplotype is equally predominant in Longhaired Whippet, Silken Windhound, Shetland sheepdog and Australian sheepdog, but not in Collie, which suggests that the mutation is coming from the Greyhound line (Geyer J. *et al*., 2007). In this study dog breeds are seen as dynamic populations that have historically experienced admixture, introgression, and genetic isolation. The presence of MDR1 in the Sighthounds breeds supports this theory, and confirms that different regions of the canine genome have different evolutionary histories (Neff M. et al., 2004). Therefore, breed phylogeny is perhaps less relevant than the phylogeny of individual traits.

2.2.6.3 Autosomal recessive transmission

The canine genome is composed by 78 chromosomes, forming 39 pairs, and about 20,000 genes. A gene at the same locus on a matching chromosome is called an allele and each individual has 2 alleles per genetic position, each one, received from one parent. The

individuals can be homozygous (equal alleles) or heterozygous (different alleles) and, the last ones, the mutant allele can cause or not a disease, conform it is a recessive or dominant allele. In recessive cases, the offspring requires 2 copies of a recessive mutated gene from both parents to develop disease, however, if they receive just one copy, they will carry but not develop the disease and are able to transmit it to the next generation. If both parents are carriers, there is a 1 in 4 chance that the offspring will inherit both mutated gene copies and develop the disease. However, if one parent is a carrier and the other is homozygous recessive, there is 50% chance that the offspring will, as well, be affected with the disease. In a dominant way, only one copy of the mutant allele is necessary to express the disease. Other cases show another possibility, an incomplete dominance or autosomal dominant disorders with incomplete penetrance, which causes a variable expressivity in the trait (Ostrander E. & Robert K., 2005).

Schinkel A. *et al*., (1994) used the mouse strain CF1 and proved that the mutation follows the Mendelian laws with the transmission occurring on an autosomal recessive way. Only homozygous individuals, for the mutated allele, were sensitive to ivermectin.

Similarly in Collie, studies show that only homozygous dogs are sensitive to the drug and the percentages of homozygous, wild-type and mutants, and heterozygous dogs are consistent with this type of transmission. Although, it happens that some heterozygous dogs appear particularly sensitive to the vincristine and vinblastine action, which make us think about the possibility that Pgp in this animals is not present in a sufficient quantity or that some substrates or other proteins may be involved. In some cases, it appears that ivermectin has the same toxic doses as for homozygous dogs.

Table 10: Advices for therapeutic use of some drugs in heterozygous and homozygous mutant dogs (adapted from: "Veterinary Clinical Pharmacology Lab" of Washington State University).

Molecules/ Genotype	**Acepromazine, butorphanol, vincristine, vinblastine and doxorubicin.**	**Ivermectin, doramectine, selamectine, moxidectine and mylbemecyme.**	**Emodepside and loperamide**	**Cyclosporin, Digoxin and Doxycicline**
Heterozygous	Reduce 25%	Safe if used for heartworm prevention at the manufacturer recommended dose. Higher doses (10-20 folds) can cause neurological toxicity.	Do not use. Therapeutic doses results in neurotoxicity.	Not alter the dose. Only therapeutic drug monitoring.
Homozygous mutant	Reduce 30-50%	Safe if used for heartworm prevention at the manufacturer recommended dose. Higher doses (10-20 folds) cause neurological toxicity.	Do not use. Therapeutic doses results in neurotoxicity.	Not alter the dose. Only therapeutic drug monitoring.

2.2.6.4 Prevalence in each breed and impact on their selection

Numerous studies in diverse countries were performed to find the frequency of the mutant allele of MDR1m gene in different populations of the Collie breed. The results are resumed in table 11.

Table 11: Frequency (%) and average of MDR1m gene in different populations of Collies, from distinct countries (adapted from: Geyer J. et al 2005; Hugnet C., Bentjen S., Mealey K., 2004; Kawabata A., Momoi Y., Inoue-Murayama M. & Iwasaki T., 2005; Mealey K., Bentjen S. & Waiting D., 2002; Mealey K., Munyard K. & Bentjen S., 2005; Neff M. *et al.*, 2004).

Percentage/Country	Homozygous (wildtype)	Heterozygous	Homozygous (mutant)
Australia	12	64	24
Belgic	14	57	29
U.S.A.	22	42	35
France	20	32	48
German	24	43	33
Japan	25	33	42
Average	**19,5**	**45,2**	**35,2**

Other breeds have also been studied and their percentages in several populations of different countries analyzed. To construct table 12 data resulting from different studies were used and the average of the results calculated, and we can see that Collies are not the only breed with a high prevalence of this mutant gene.

Table 12: The average frequency (%) of MDR1m gene in different breeds (adapted from: Neff M. et al., 2004; Cunningham F. et al., 2010).

Percentage/Country	Homozygous (wildtype)	Heterozygous	Homozygous (mutant)
Australian Shepherd dog (standard and miniature)	60, 1	37,2	2, 7
Border collie	99,1	0.6	0,3
English shepherd dog	85,7	14,3	0
German Shepherd dog	90,0	8	2
Longhaired whippet	15,7	51,7	32,6
McNab	68,6	28,6	2,8
Old English sheepdog	92,7	7,3	0
Shetland sheepdog	84,2	14,7	1,1
Silken windhound	65,5	33,3	1,2
White Swiss shepherd dog	76,2	21,5	2,3

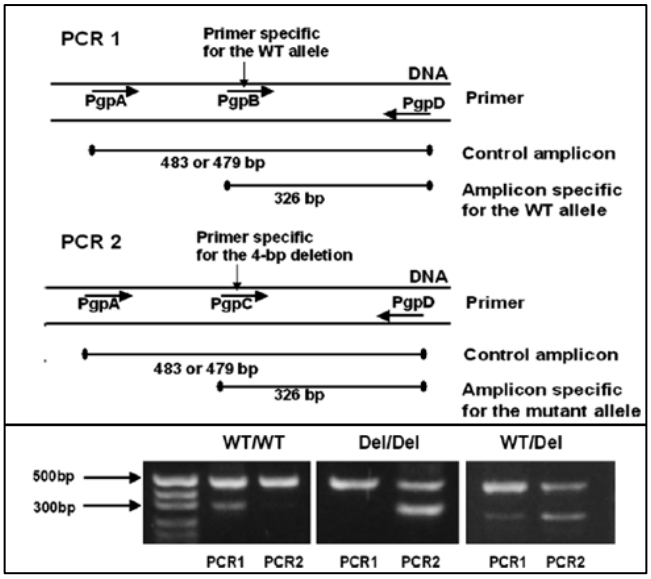
2.2.6.5 Genetic screening tests

From previously data it can be concluded that a significant percentage of Collies are homozygous for the mutated allele and 10 other breeds are also affected, however, in lower proportions. The study of Pgp and clinical cases verified important medical consequences, sometimes fatal, when applied to certain veterinary commonly molecules. These consequences demonstrate the need of individual genetic screening tests to adapt the therapeutic according to the individual sensitivity. It is not logical that a patient undergoes the entire therapeutic arsenal, just because some individuals from that breed are known to be sensible to some molecules (Dowling, P., 2006; Lafon M., 2005).

Initially, genotyping Collies for MDR1 gene mutation was based on DNA sequencing, a technique relatively expensive and time consuming, which required specialized laboratory equipment. Later, several team researches have used the application of PCR method for genotype and amplification of one DNA fragment containing the possible deletion. One same principle was used: amplify DNA fragments containing the suspect deletion fragment to compare and separate by polyacrylamide gel electrophoresis and, then, directly visualize it after staining with ethidium bromide. Since both types of fragments will differ only by 4 bp, the primers were chosen to amplify short sequences (Mealey K. *et al*., 2001).

The study of Roulet *et al*., (2003) used mouth and colon cells, from Collies and Beagles, and genotyped this dogs for the mutant allele and wild-type allele. Despite the results obtained during this study are identical to those obtained by sequencing, the results are not reliable, perhaps because of bacterial contamination and possible cross-amplification of bacterial sequences of ABC transporters. Furthermore, it seems that the length of the amplified fragments did not correspond to the expected, comparing to the primers used. Another team used blood samples to avoid the possible contaminations. The primers used allowed the detection of an additional "heteroduplex" band in the cases with a heterozygous mutation (Geyer J. *et al*., 2005). Although both studies results are identical to those obtained by sequencing, several authors do not trust this method by itself requiring sequencing confirmation, based on the fact that it is difficult to differentiate two PCR products which differ only in 4 base pairs. Later, one third team decided to develop an allele-specific PCR method and also improved two PCRs with different primers, PCR1 for detection of wild-type allele and PCR2 for mutate allele. Each PCR generates the amplification of 326 base pairs and independently of the presence or absence of the mutated allele, a control amplicon (a piece of DNA that has been synthesized using amplification techniques) is present with PCR1 and PCR2.

Figure 10: Design of the allele-specific PCR (From: Baars C. et al., 2007).



Description:

Above: the assembly of the two forward primers and one reverse primer are shown schematically for PCR1 and PCR2. PCR1 was designed to detect the wild type MDR1 allele and PCR2 to detect the mutant MDR1 allele.

Under: Results of allele-specific PCR testing in dogs with different MDR1 genotypes.

More than 55 dogs were sequenced and tested with this method to confirm the identical and reproductive results, which occurred. The samples were blood and buccal swabs and no difference in reliability was noted between the two types of samples, which allow the use of a minimally invasive genetic test (Baars C., Leeb T., Klopmann T., Tipold A., Potschka H., 2007). Then, a fast, economic and reliable test appears, permitting to test any dog for the MDR mutation.

2.2.6.6 Breeding Strategies

The knowledge of genetic diseases and recognized affected animals or disease carriers is important in reducing inherited disorders and to draw a reproduction strategy. In recessive autosomal diseases, when the frequency of carriers is low (below < 5%), the best option is to remove all of them from the breeding population. But, when a significant proportion of carriers are present, the overall health situation may be exacerbated by removing all the carriers from the breeding population as it would constrict the gene pool for the future. Probably, the disease being tested might be eradicated, but will increase the proportion of the breed carrying other deleterious mutations. So, when the initial carrier frequency is high like in the Collies and other related breeds, relative to the MDR1m, the slow removal of the disease mutation over many generations will maintain more genetic diversity in the breed (Karriker M., 2007). Hence, DNA tests are useful for dog owners, to know how to bring up their dogs properly; veterinarians, to help in diagnostic and prescription of therapy and dog

breeders and potential dog owners, to avoid problems when choosing a pet and to avoid defective genes in their lines (Fleischer S. *et al*., 2008).

2.3 Therapeutic approach for treatment of avermectins poisoning

Usually, cases of avermectins dogs intoxications results mostly from accidental expositions, exacerbated by the presence of MDR1m allele. It is also common when the drug is given by the owner, normally destined to other species (off-label use), or when the veterinary is not alert for the breed predisposition sensibility to the drug. Sometimes the intoxication is not clear, no pathognomonic signs occur. Through an exhaustive anamnesis to understand all possible exposures in the precedent week, taking into account that the dog belongs to the 11 breeds with mutation prevalence and connecting the several clinical signs showed by the patient, it is possible to create a link between exposure to the molecule and the presented intoxication (Thisse A., 1995).

2.3.1 A general approach for cases of poisoning

In a general approach the basic toxicological principle "treat the patient and not the toxicant" is followed. Most intoxicated patients recover with close monitoring, appropriate symptomatic intervention, and good nursing care. The treatment for intoxication includes 3 basic principles.

Prevention of Further Absorption: topically applied toxicants usually can be removed by thorough washing with soap and water; clipping of the hair or wool may be necessary. Emesis is of value in dogs if done within a few hours of ingestion but is contraindicated when the swallowing reflex is absent, when the animal is convulsing or risk of aspiration pneumonia is imminent. Oral emetics include syrup of ipecac (10-20 mL, PO in dogs), and hydrogen peroxide (2 mL/kg, PO). Apomorphine can be used in dogs, via parenteral, at a dose of 0.05-0.1 mg/kg. On unconscious or anesthetized animal gastric lavage is done with the largest bore stomach tube possible and it is essential the use of an endotracheal tube. Cathartics and laxatives could be indicated in some cases for more quick elimination of the toxicant from the GI tract. A gastrotomy is possible to be required when lavage techniques are insufficient. Occasionally the poison cannot be physically removed. However, several substances administered orally can adsorb it and prevent its absorption from the alimentary tract. Activated charcoal (1-2 g/kg) is effective in adsorbing an extensive diversity of compounds and typically it is the adsorbant and detoxicant of choice when poisoning is suspected.

Supportive Therapy: Frequently the supportive care is needed while the toxicant is metabolized and eliminated. The type of support required depends on the animal's clinical condition and may consist in control of convulsive seizures, maintenance of respiration, treatment for shock, correction of electrolyte and fluid loss, control of cardiac dysfunction, and alleviation of pain.

Specific Antidotes: Antidotes (when known) are listed for each toxicant. Some form complexes with the toxicant, others block or compete for receptor sites and a few affect the metabolism of the toxicant (Gallagher A. & Noftsinger M., 2008; Heit J., Tranquilli W., Parker A., Paul A. & Sisson D. 1989; King, L. & Hammond R., 1999; Plumb D., 2001).

2.3.1.1 Different approaches to avermectins intoxication

Especially to avermectins, therapy should focus on gastrointestinal decontamination with activated charcoal and sorbitol cathartics (Birchard S. & Sherding R., 2008). It is suggested that repeated applications of activated charcoal may prevent enterohepatic recycling of ivermectin. This is not certain but it seems appropriate to try this approach until proven ineffective. Supportive therapy may be prolonged in dogs (days to weeks), consisting of intravenous fluids, padding for the comatose animal, frequent turning of affected animals to prevent pressure sores, and careful monitoring. There is no effective antidote to avermectins, although, different protocols have been proposed (Commission Nationale de Pharmacovigilance Veterinaire [CNPV], 2005).

At the beginning, there were some reports using picrotoxin as an antidote to reverse the effects of an ivermectin toxicosis. This drug acts as a potent noncompetitive GABA antagonist and causes an increase in the excitability of neurons in the CNS, as leads to convulsions and picrotoxin has a narrow margin of safety, its use is not recommended (American Board of Veterinary Toxicology [ABVT], 2012).

Physostigmine is an uncharged, reversible inhibitor of acetylcholinesterase that can penetrate the BBB. This drug has been shown to have some effect in comatose animals, probably due to an increased concentration of acetylcholine in affected neurons. The comatose animal may exhibit a transient increase in mental alertness. This can be helpful to the veterinarian to confirm the diagnosis. Adverse effects associated with physostigmine include convulsions, cholinergic crisis as ptyalism, bradycardia, and dyspnea, variable levels of consciousness and possible death. Physostigmine is quickly metabolized and requires repeated administration (every 30 - 60 minutes) to preserve effects. Due to its clinical limitations and possible adverse side effects, physostigmine administration as an analeptic is not recommended, except to persuade owners to not select euthanasia, (Tranquilli W., Paul A., Seward R., Todd K. & Dipietro J., 1987).

Sarmazenil, a competitive antagonist at the benzodiazepine binding site of the GABA, was used to help reversion of the clinical signs of moxidectin intoxication in one foal. The patient presented an improvement after this treatment but it was not possible to confirm the direct

effect of sarmazenil because recovery time was similar to other colts who did not receive this treatment (Müller J., Feige K., Kästner S. & Naegeli H., 2005).

2.3.2 Intravenous lipid emulsion: a potential novel antidote

Intravenous lipid emulsion (ILE), also named as IV fat emulsions, has been reported as an antidote in cases of local anesthetic and other lipophilic drug toxicosis. Clinical use of lipid emulsions as part of a parenteral nutrition formulation began during the 1960s with the production of soybean-oil-based formulations.

Later, between 1970 and 1980, several studies evaluated the effects of ILE in the pharmacokinetics of chlorpromazine and cyclosporine in rabbits and phenytoin in rats. One study demonstrated that the infusion of a lipid emulsion shifts the dose–response of bupivacaine induced cardiac arrest in rats. This emphasized the potential favorable effects of ILE in the treatment of local anesthetic toxicosis.

In human medicine, the administration of ILE is usually reserved for severe toxicosis and lifethreatening conditions and when common therapies have failed to improve physiological parameters. In veterinary medicine its use is justified for intoxications related with high morbidity, for which traditional therapies (including ventilator management) have failed or are cost prohibitive. So, the administration of ILE is generally initiated earlier in the course of therapy in symptomatic patients. Common to both, human and veterinary medicine, is the notion that ILE therapy is generally safe. Response to ILE therapy has resulted in insignificant improvements or complete resolution of clinical signs associated with toxicosis. The variation in response is thought to be related to the lipid solubility of the toxin in question.

2.3.2.1 Action mechanism

The exact ILE action mechanisms that allow to increase the rate of recovery and increases conventional resuscitation efforts in various cases of lipophilic drug intoxications is unknown and diverse theories are considered:

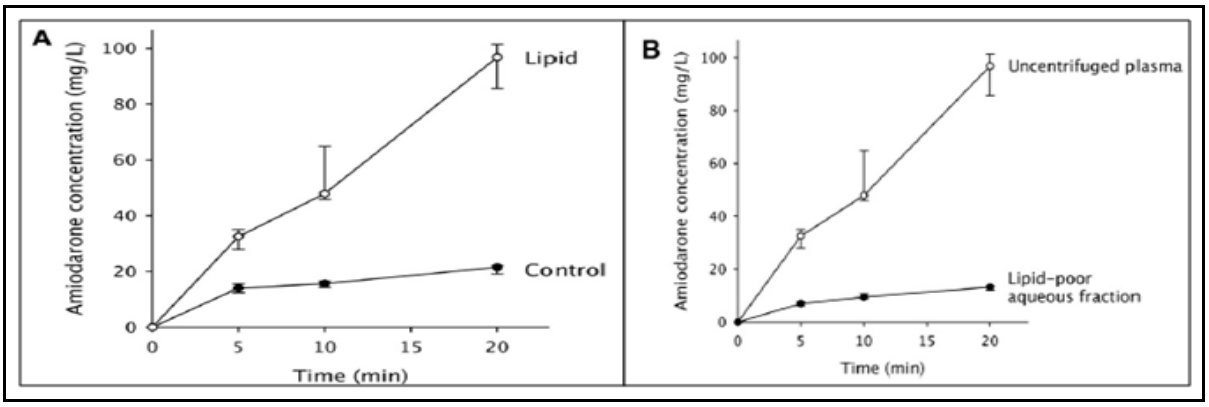
The beneficial effects of ILE therapy may be linked to improvements in cardiac function (myocardial performance) through either the direct benefit of lipids on the myocardium or the reversal of cardiovascular dysfunction caused by the specific toxicant. These effects can be related with different mechanisms such as the use of free fat acids (FFA) as an energy source by the myocardium, an increase in intracellular calcium, alfa-adrenergic receptor mediated increased vasopressor effect, and the reduction of nitrous oxide and insulin-induced vasodilatation by ILE. It is believed that ILE can improve cardiac performance by provision of energy substrates to the myocytes in the form of FFA. Endogenous FFA is used as the

preferred substrate for myocardial energy production in the resting myocardium (Fernandez A. *et al*, 2011). Velde V. *et al* (1996) investigated the effects of increasing plasma triglyceride concentration in the stunned canine myocardium and verified an improvement in the functional recovery from myocardial ischemia when high doses of lipids were administered during the post-ischemic reperfusion phase.

Myocardial performance may also be improved as a function of increased intracellular calcium concentration. Studies performed on isolated cardiac tissue demonstrated that increased availability of FFA stimulates the activation of voltage-gated calcium channels in the myocardium, increases cytosolic calcium concentrations and increases cardiac function. An acute myocardial ischemia causes an increase in intracellular calcium concentrations which can give origin to injurious effects. However, this increase can improve cardiac function in cases of myocardial dysfunction secondary to calcium channel blocker toxicosis (Rothschild L., Bern S., Oswald S. & Weinberg G., 2010).

The beneficial effects of ILE in cases of lipophilic drug toxicosis can be explained by the artificial creation of a lipid compartment in the intravascular space (drug sequestration or "lipid sink") where the lipophilic compounds are sequestrated. Technically, this results in a higher concentration of drug or toxicant in the plasma with less free drug available to the tissues, therefore decreasing its toxic effects. This 'lipid sink' theory has also been supported with toxicosis involving chlorpromazine, bupropion, mepivacaine, and bupivacaine (Fernandez A. et al, 2011). Studies performed in pigs with amiodarone had as objectives to investigate to what extent amiodarone is sequestered by intravenously administered lipid emulsion in plasma and whether the lipid emulsion inhibits amiodarone-induced hypotension. Plasma amiodarone concentration and mean arterial blood pressure of 20 anesthetized pigs were evaluated after administration of a bolus injection of olive/soybean oil–based 20% lipid emulsion or Ringer's acetate solution, followed by a continuous infusion with amiodarone hydrochloride in both groups. Plasma amiodarone concentration in the lipid group increased more steeply during the amiodarone infusion than in the control group. After separation of lipids from plasma by differential centrifugation, less amiodarone was contained in the lipid-poor aqueous fraction (figure 11). In the lipid group, mean arterial blood pressure was not altered during the continuous amiodarone infusion.

Figure 11: Amiodarone concentrations (mg/L) in uncentrifuged plasma of the lipid and control groups and in plasma of the lipid group (from: Niiya T., Litonius E., Petäjä L., Neuvonen P. & Rosenberg P., 2010).



Description: A) Time course of the plasma amiodarone concentration was significantly different between groups. B) The amiodarone concentration in the lipid-rich uncentrifuged plasma was significantly higher than in the lipid-poor aqueous fraction.

These results point to the "lipid sink" theory, where amiodarone was sequestered to a great extent by the intravenously administered lipids in plasma, which completely prevented the decrease in arterial blood pressure caused by amiodarone infusion (Niiya T., 2010).

2.3.2.2 Proposed protocols

Given the difficulties to determining the mode of action, the proposed protocols in human medicine are very empirical. The most used protocol included the administration of a IV bolus of 1.5 mL/kg bw, possibly followed by a maintenance infusion 0.2-0.5 mL/kg/min, depending on the animal clinical response (Pritchard J., 2010).

2.3.2.3 Adverse effects

Adverse effects of ILE are unusual and they can come from a contamination of the lipid product or from a directly reaction to the emulsion. For nutrient-rich products, such as lipid emulsions, contamination is a particular concern. Due to an incorrect management or non-sterile technique, the lipid product can suffer a microbial contamination, resulting in a systemic infection and venous irritation, with subsequent thrombophlebitis. However, this rarely occurs when ILE is infused alone. Adverse effects of ILE may also be due to direct reaction to the emulsion, which results in an acute adverse pyrogenic reaction or "colloid" reaction. Clinical reactions, occurring in less than 1% of human cases, including "anaphylactoid-like signs", can occur within 20 minutes of administration. Signs comprise fever, nausea, vomiting, dyspnea, tachypnea, cyanosis, arrhythmias, hypotension and

cardiovascular collapse. Equally, allergic reactions can occur due to the egg phospholipid or to the soybean oil component. Delayed or subacute reactions to ILE may also occur and are commonly referred to as "fat overload syndrome" (FOS), resulting from the excessive volumes or high administration rates, exceeding the endogenous lipid clearance mechanisms. In Humans, FOS can result in fat embolism, hyperlipidemia, hepatomegaly, icterus, splenomegaly, thrombocytopenia, increased clotting times and hemolysis.

Other type of reports covers neurological complications, associated with chronic administration of lipids. Multifocal deficits and focal seizures have been observed in humans and histological evaluation of brain tissue shows perivascular edema and neutral lipid in the pericytes of many capillaries as well as intra-arteriolar and capillary neutral lipid emboli. Relatively to the respiratory tract, administration of a 20% ILE in critically ill, septic patients, and in those suffering from acute respiratory distress syndrome resulted in an increase in the mean pulmonary artery pressure, augmented venous admixture, reduced partial pressure of arterial oxygen to fraction of inspired oxygen level, increased alveolar/arterial partial pressure of oxygen gradient, and intrapulmonary shunting in patients. Changes affecting bronchoalveolar fluid were seen, suggesting deterioration of the blood-gas barrier permeability, inflammation of lung tissue, and changes in alveolar surfactant characteristics, though, only in patients with acute respiratory distress syndrome. This indicates a higher risk of developing temporary changes in pulmonary function and oxygenation parameters in these patients.

The occurrence of adverse effects, after ILE administration, in patients with infectious pulmonary disease or chronic obstructive pulmonary disease is rare.

The establishment of lipemia and hypertriglyceridemia, the last one, associated with an increased risk of cardiovascular disease and pancreatitis. However, a cause and effect relationship between transient hypertriglyceridemia and pancreatitis has not been confirmed already (Fernandez A. *et al*, 2011).

Believing that specific formulation of the ILE itself can also have variable physiologic effects or adverse events, Velde V. *et al* (1996) performed a study to compare the hemodynamic effects of 3 different ILE preparations in dogs. Treatments consisted of Intralipid 20%, Medialipide 20%, or 20% omega-3 polyunsaturated fatty acids (PUFA) emulsion administered at 7 mL/kg bw. The results with Intralipid lead to an insignificant increase in heart rate and a transient decrease in arterial pH. Treatment with Medialipid 20% and the omega-3 PUFA emulsion caused a reduction in myocardial contractile performance. In cardiovascular compromised patients, the use of these 2 emulsions should be cautiously considered.

2.3.3 Clinical applications

The successful treatment of canine moxidectin intoxication with the novel therapy of ILE administration was described (Crandell D. & Weinberg G., 2009). The clinical case involves a young Jack Russell Terrier female, presented with acute onset of seizures followed by paralysis and coma. Moxidectin toxicity was later confirmed, after suspected exposure to an equine formulation. A first supportive treatment was applied and later an emulsion of 20% soybean oil in water was administered as IV bolus for 4 hours, beginning 10 hours after exposure, and was administered again for 30 minutes beginning 25.5 hours post-exposure. Insignificant improvement was seen after the first dose, although, a large improvement was noted within 30 minutes of the second dose. The puppy's neurologic status returned to normal within 6 hours of the second administration, with no relapses (Crandell D. & Weinberg G., 2009). Another case refers a 2-year-old female Border collie treated with ILE after ingesting 6 mg/kg of an equine ivermectin anthelmintic paste 8 hours prior to examination (Clarke D., *et al*. 2011).The dog had stable cardiovascular signs but had diffuse muscle tremors and was hyperthermic. Neurologic evaluation indicated ataxic and mydriasis with bilaterally absent menace responses and pupillary light reflexes. Additionally to supportive care IV fluid therapy and cardiovascular, respiratory and neurologic monitoring, ILE was given to the dog. An initial bolus of a 20% sterile lipid solution was administered over 10 minutes, followed by a constant rate infusion over 60 minutes that was administered twice to treat clinical signs of ivermectin toxicosis. The dog had great improvements and was discharged from the hospital 48 hours after admission. Further diagnostic evaluation revealed that this dog was unaffected by the MDR mutation (Clarke D., *et al*. 2011).

Numerous studies evaluate the ILE treatment in several drugs intoxications for example with local anesthetics, clomipramine, verapamil, haloperidol, amlodipine, propranolol, moxidectin and others. However, to several authors, the treatment seems to be more effective and useful in avermectins intoxication (Clarke D., Lee J., Murphy L. & Reineke E., 2011).

3 Practical approach to pharmacovigilance and pharmacogenetics contexts

3.1 Analysis of data from the Sentinel-Vet software

As referred above, the French veterinary pharmacovigilance system, at CPVL, is responsible for collecting data of ADRs. CPVL is supported by the Sentinel-Vet software, specific for veterinary pharmacovigilance. All the phone calls or written reports are entered into this database, in English. The database complies with Eudravigilance requirements: it uses published list regarding species, breeds and clinical signs (Veddra). Every suspected drug is assigned causality using the ABON classification (Pineau X., personal communication, 16 September 2012).

3.1.1 Objectives

This part pursues the purpose of analyze several ADRs using the Sentinel-vet database. Three molecules were chosen for analysis to verify if exists an increased number of adverse reactions in some dog breeds with the MDR1 mutant gene presented in their population.

3.1.2 Material and methods

The Sentinel-vet software allows the user to select the data needed through filters. Through the literature and after studying different variables the clinical cases collected were notified during the period between 2005 and 2011. All animals belong to the canine species and notifications with the classification "N", asymptomatic exposures or general information were not taken into account. The research was done using the active substance as the keyword and not any specific pharmaceutical medicine. The clinical data was later recovered and worked on in an Excel Sheet. Sometimes the data was not completely fulfilled, which made it necessary to consult the report in a paper form stored in the center.

The molecules of study are: ivermectin, because it was the first molecule where this problem arose and it is the reason why the drug sensitivity of certain breeds was associated to a MDR1 gene mutation. Also, these data can reveal if veterinarians and pet owners are more alert to this problem; loperamide, because it is referred by the "Veterinary Clinical Pharmacology Laboratory"- Washington State University- as not safety in dogs and inadvisable to use in homozygous mutants and carriers in therapeutic doses. Also, as it can be seen in the table 13, this drug occupies the 56th place of the most reported drugs in dogs to CPVL, and the veterinarians do not seem alert to this problem despite LOPERAL, which active substance is loperamide, has on the leaflet "not to use in Collies and related breeds"; emodepside, because there are only a few studies about this molecule, and it is placed in the 17rd of the most

reported drugs in dogs to CPVL. However, its SPC refers the special caution when applied in breeds with higher sensitivity.

More, despite the White Swiss shepherd dog and German shepherd dog being different breeds, they are in the same category due their closeness.

3.1.3 Results and discussion

In first place, CPVL received an impressive total of 5529 reports during the referred period, as we can see in table 13. The position of the 3 molecules chosen is highlighted relatively to the rest of the reported molecules.

Table 13: Resume of the Sentinel-vet data in order of the most reported substances concerning dogs (n = 5529, CPVL, 2005-2011).

Substances	Number	Percentage (%)
15º Ivermectin	170[*a]	3.1
17º Emodepside	154[*b]	2.8
56º Loperamide	48[*c]	0,9
Total of reports	5529	100

*a Includes 4 "N" reports.
*b Includes 4 "N" reports.
*c Includes 2 "N" reports.

3.1.3.1 Ivermectin

The following table shows the main symptoms referred to CPVL when an ADR related with the use of ivermectin occurred (cases of causality N-unlikely removed, symptoms expressed as "Preferred Term" of the Veddra terminology).

Table 14: Frequency of reported symptoms in dogs after an ADR related with ivermectin use (n= 166, CPVL, 2005-2011).

Symptom (Preferred Term)	No of occurrences	Frequency of occurrences (≥6%)
1) Ataxia	73	44
2) Muscle tremor	49	29,5
3) Mydriasis	42	25,3
4) Lethargy	40	24,1
5) Death	25	15,1
6) Blindness	25	15,1
7) Emesis	20	12
8) Convulsion	20	12
9) Coma	17	10,2
10) Hypersalivation	15	9
11) Paresis	14	8,4
12) Hiperactivity	14	8,4
13) Paralysis	13	7,8
14) Impaired vision	13	7,8
15) Anorexia	13	7,8
16) Amaurosis	13	7,8
17) Hyperaesthesia	11	6,6
18) Other signs	135	81

The symptoms involve mainly the nervous system and are considered serious. The "mortality" appears in the 5th place of the most common symptoms, making it a concerning situation.

One important question to answer is if sensitive breeds represent a great percentage of the total of ADRs reported above to CPVL.

Figure 12: Percentages of ADRs related with ivermectin reports by breeds (n= 166, CPVL, 2005-2011).

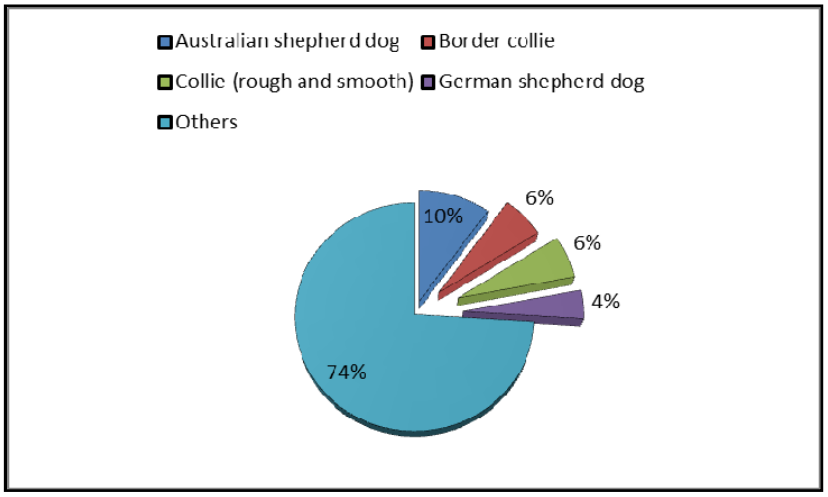
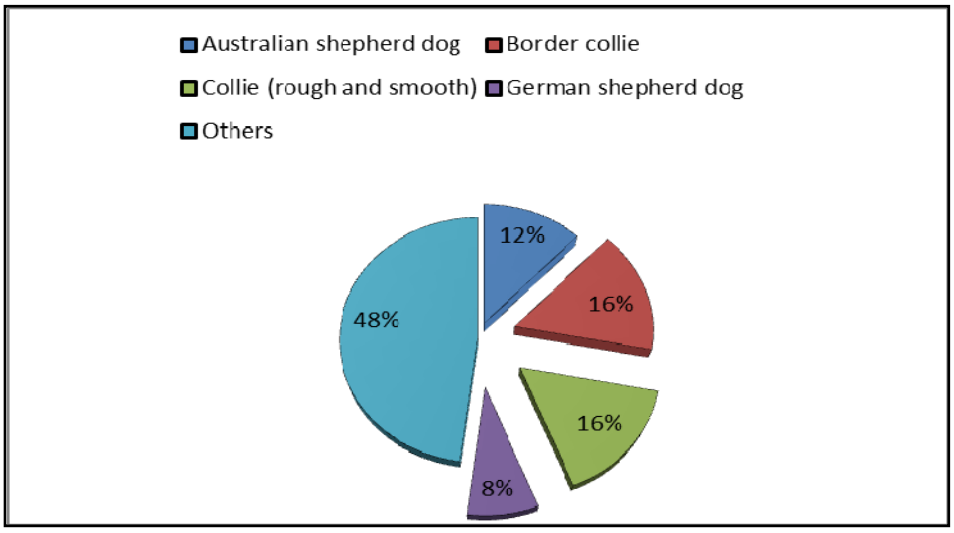


Figure 12 shows that sensitive breeds represent only 26% of the total canine population with ADRs reported after ivermectin use. This can indicate a better knowledge by veterinarian and pet owners, because the SPC indicates, since 2001, that its use is not advisable, especially in these breeds. However, the data can be camouflaged because there are few recommendations to administer ivermectin in dogs on label use, which origins a large number of reactions not related with any sort of mutation.

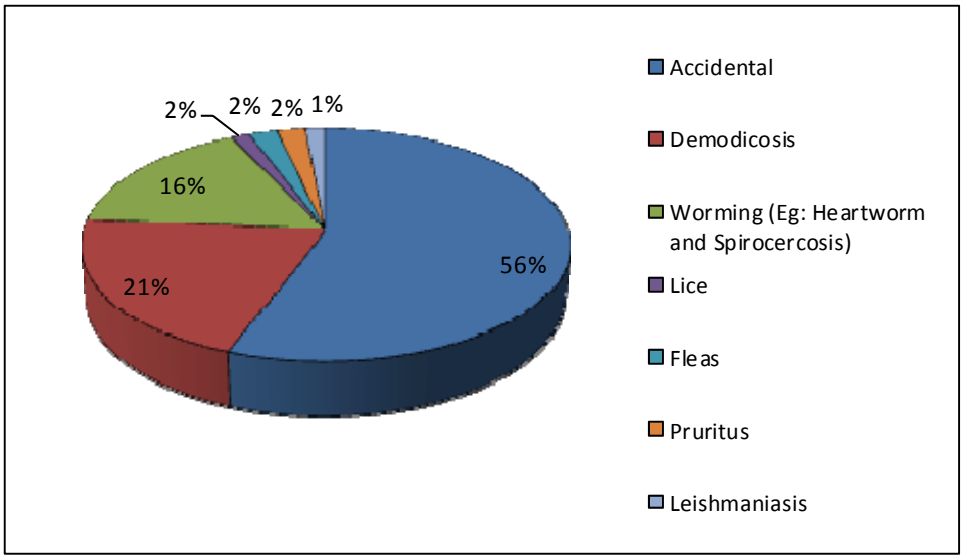
However, theoretically, it will be expected that this category of breeds, with the mutant gene, have more serious reactions than others, and it is important the observation of the figure 13. This graphic specifies that 52% of the serious reaction includes four breeds of dogs considered carriers of the mutant allele.

Figure 13: Percentages of serious ADRs related with ivermectin reports by breeds, (n=25, CPVL, 2005-2011).



The reason why ivermectin is given to dogs is an important aspect that should be investigated and the results are presented in figure 14.

Figure 14: Reasons of ivermectin administration according the reports (n= 166, CPVL, 2005-2011).

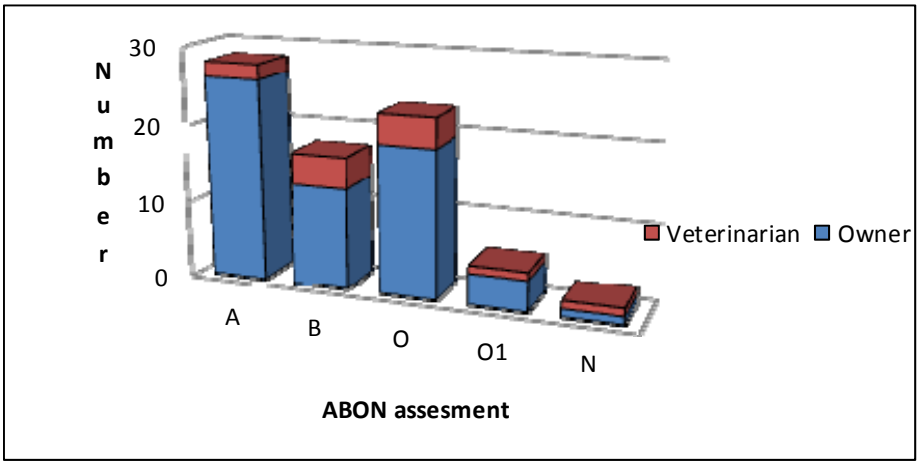


As can be appreciated, the use of this molecule is essentially linked with accidental exposure. Observing case by case in the Sentinel-vet software, it can be concluded that most accidental situations, referred above, occurred with dogs ingesting the horse dewormer, which indicates that the owners must be alerted about this, unfortunately, common situation.

The recommended use of ivermectin in dogs, to treat *Dirofilaria immitis,* appears only on the 3rd place and veterinarians still prescribe ivermectin as a treatment for demodicosis, 2nd place, even in off-label use.

Removing the accidental cases, an analysis can be made relating the person who administered the drug, veterinarian or pet owner, with the causality assessment given. The figure 15 shows that most reported ADRs were assessment with "A" category and owners are the responsible by the animal exposure. This is logical since ivermectin is prescribed by Veterinarians but the daily admistration is made by owners which may lead to dose mistakes.

Figure 15: Numbers of ADRs related with ivermectin according its causality assessment and person which administered (n= 74, CPVL, 2005-2011).



3.1.3.2 Loperamide

On table 15 the frequency of symptoms reported in dogs after loperamide exposure are presented (cases of causality N-unlikely removed, symptoms expressed as "Preferred Term" of the Veddra terminology).

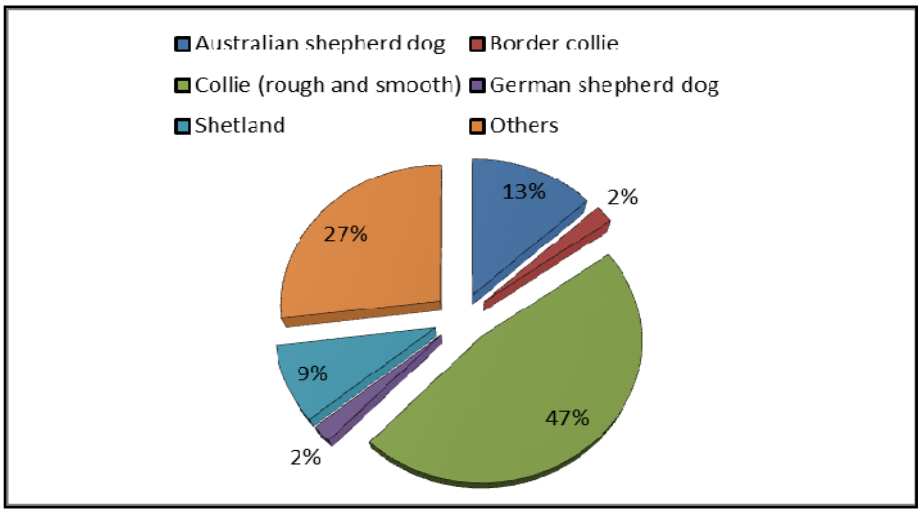
Table 15: Frequency of reported symptoms in dogs after an ADR related with loperamide use (n=45, CPVL, 2005-2011).

Symptom (Preferred Term).	No of occurences	Frequency of occurrences (≥6%)
1) Lethargy	18	40
2) Ataxia	14	31,1
3) Anorexia	10	22,2
4) Vocalisation	9	20
5) Emesis	5	11,1
6) Diarrhoea	5	11,1
7) Paresis	4	8,9
8) Hyperactivity	4	8,9
9) Mydriasis	3	6,7
10) Internal ear disorder	3	6,7
11) Abdominal	3	6,7
12) Other signs	42	92,4
Total	120	265,8

As can be observed in cases of ADR related with loperamide administration, the most common signs are also connected with the nervous system. GI signs are also present but with less frequency. This also means an average of 2-3 signs per case.

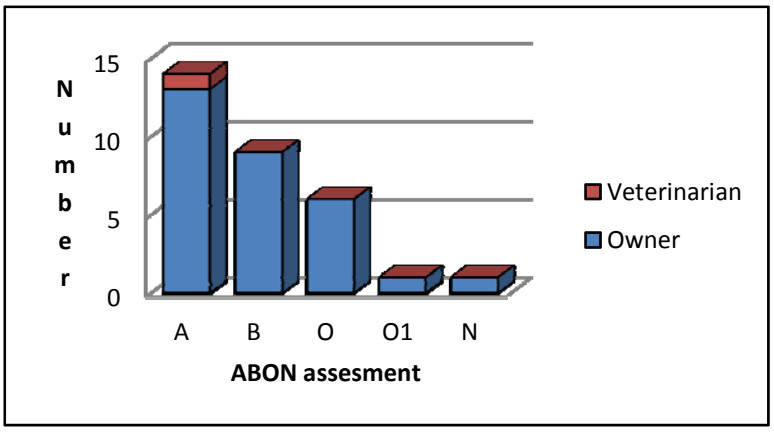
Loperamide is not usually associated with sensitive breeds but the evaluation of data provided by Sentinel-vet, made these breeds stand out. In the figure 16 it can be seen that 73% of ADRs are presented by breeds knows to be carriers of the MDR1 mutant gene. The Collie group, by itself, represents 47% of the ADRs reported to CPVL.

Figure 16: Percentages of ADRs related with loperamide reported by breeds (n=45, CPVL, 2005-2011).



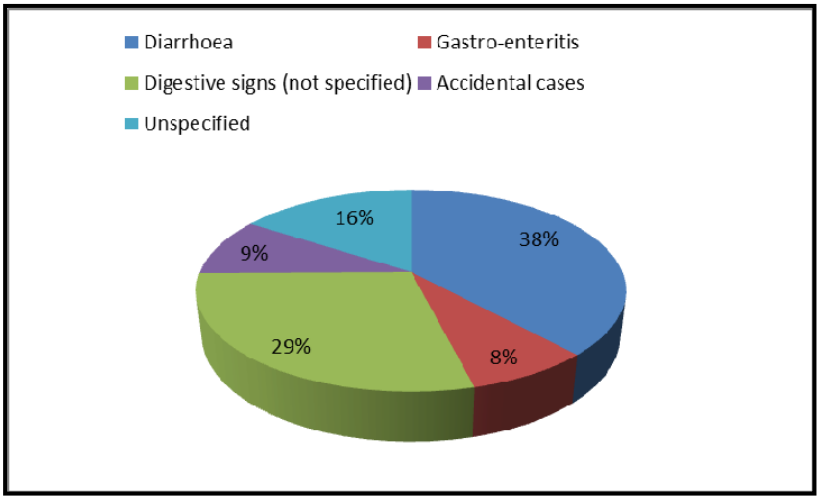
This seems to be a concerning situation, however, other questions are raised: in what conditions does this scenario take place? Is there an unfamiliarity of the veterinarian class or is it mainly given by the pet owners? In figure 17 data concerning the responsibility of drug administration are presented.

Figure 17: Numbers of ADRs related with loperamide according its causality assessment and person which administered (n= 31, CPVL, 2005-2011).



It can be concluded that the pet owners are not aware of this problem, specific sensitivity of certain breeds to loperamide. Also, loperamide belongs to a common Human medicament sold without prescription, what facilitates its administration to dogs because it is not associated with adverse symptoms. More, when the Veterinarian prescribes, usually the tablet is administered by owners. Another question is what reasons lead a person to give or a Veterinarian to prescribe loperamide?

Figure 18: Reasons for loperamide administration according the reports (n=45, CPVL, 2005-2011).



The main reason, as it was foreseen, is the administration of loperamide when the animal presents diarrhoea signs.

3.1.3.3 Emodepside

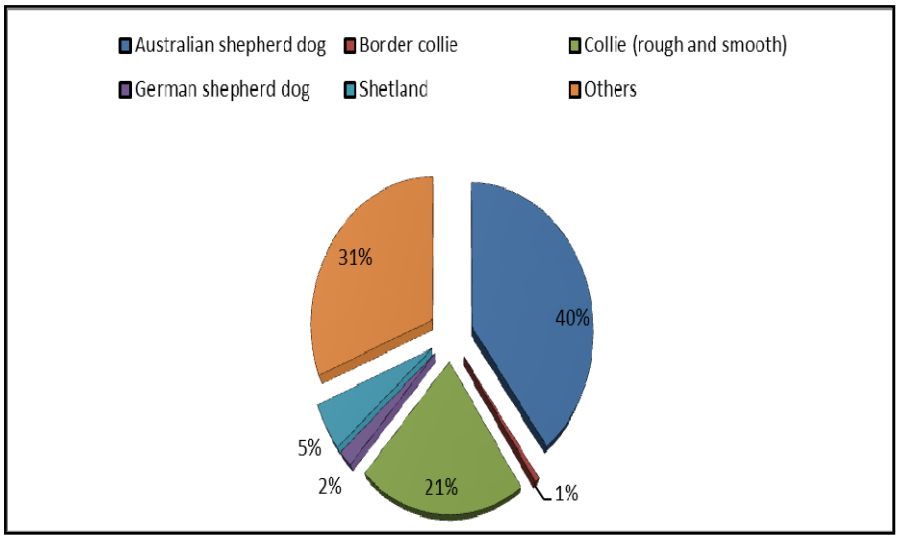
On table 16 the frequency of symptoms reported in dogs after emodepside exposure are presented (cases of causality N-unlikely removed, symptoms expressed as "Preferred Term" of the Veddra terminology).

Table 16: Frequency of reported symptoms in dogs after an ADR related with emodepside use (n= 154, CPVL, 2005-2011).

Symptom (Preferred Term)	No of occurrences.	Frequency of occurrences (%)
Muscle tremor	114	74.0
Ataxia	70	45.5
Hyperthermia	40	26.0
Hypersalivation	36	23.4
Lethargy	17	11.0
Tachypnoea	16	10.4
Convulsion	13	8.4
Myoclonus	11	7.1
Mydriasis	11	7.1
Hyperactivity	10	6.5
Emesis	10	6.5
Other signs	105	66.3
Total	453	292.2

Principal symptoms reported to CPVL after applied emodepside were muscle tremors, ataxia and hyperthermia which one, one more time, are related with the nervous system.

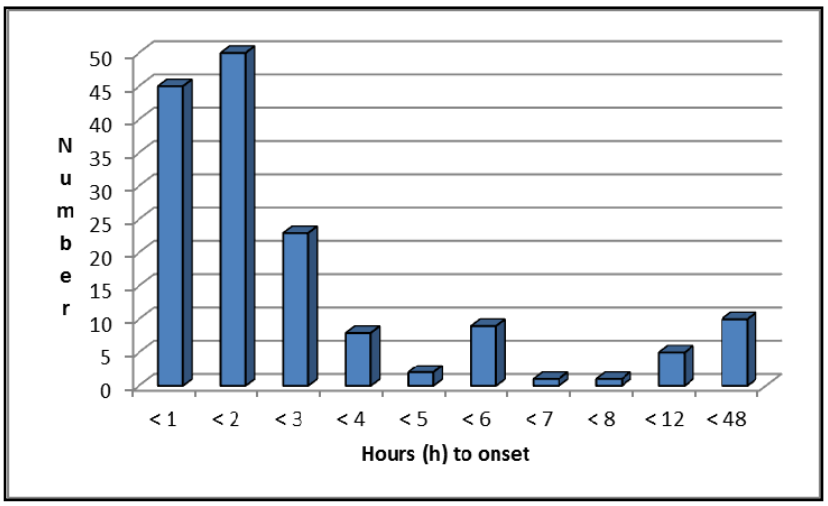
Figure 19: Percentages of serious ADRs related with emodepside reported by breeds (n=154, CPVL, 2005-2011).



The graphic above shows that Australian shepherd dogs are involved in 40% of ADRs related with emodepside reported to CPVL. The breeds known to be carriers of MDR1m gene represent 69% of the total reports, which is an important fraction. In our opinion more attention must be done to this case and eventually some measures must be taken to reduce these events.

It can be noted a strong casuality relation between drug administration and the onset of the adverse reaction. That can be observed on figure 20.

Figure 20: Time to onset the clinical signs associated with administration of emodepside (n= 154, CPVL, 2005-2011).



Observing the graphic above, the majority of the symptoms started until 3 hours after de administration of the medicament containing emodepside. This is in line with pharmacokinetics of the substance: effects are observed around plasma peak (Tmax).

3.1.4 A global analysis of data

For an integrated analysis, in the following figure ADRs related with ivermectin, loperamide and emodepside are presented together in the total of ADRs reported to CPVL concerning dogs, in the analysis period (2005-2011). If the frequency of ADRs in the total population of dogs, excluding the breeds affected by the MDR1m, shows an equal representation in the sensitivity breeds, relatively to those molecules, that will mean a irrelevance of our data. However, this did not happen and as it can be observed in the figure 21, the molecules in cause only represent 2,72% of the total of ADRs reported in the canine specie: ivermectin (1,61 %), loperamide (1,02%) and emodepside (0, 10%). These molecules certainly represent a more important fraction in the breeds in cause.

For Collies, 40,79% of the reports are related with the molecules concerned: ivermectin (7,24%), loperamide (12, 50%) and emodepside (21, 05%). More, if we consider other molecules which are subtracts of Pgp, probably these frequencies would have been even greater.

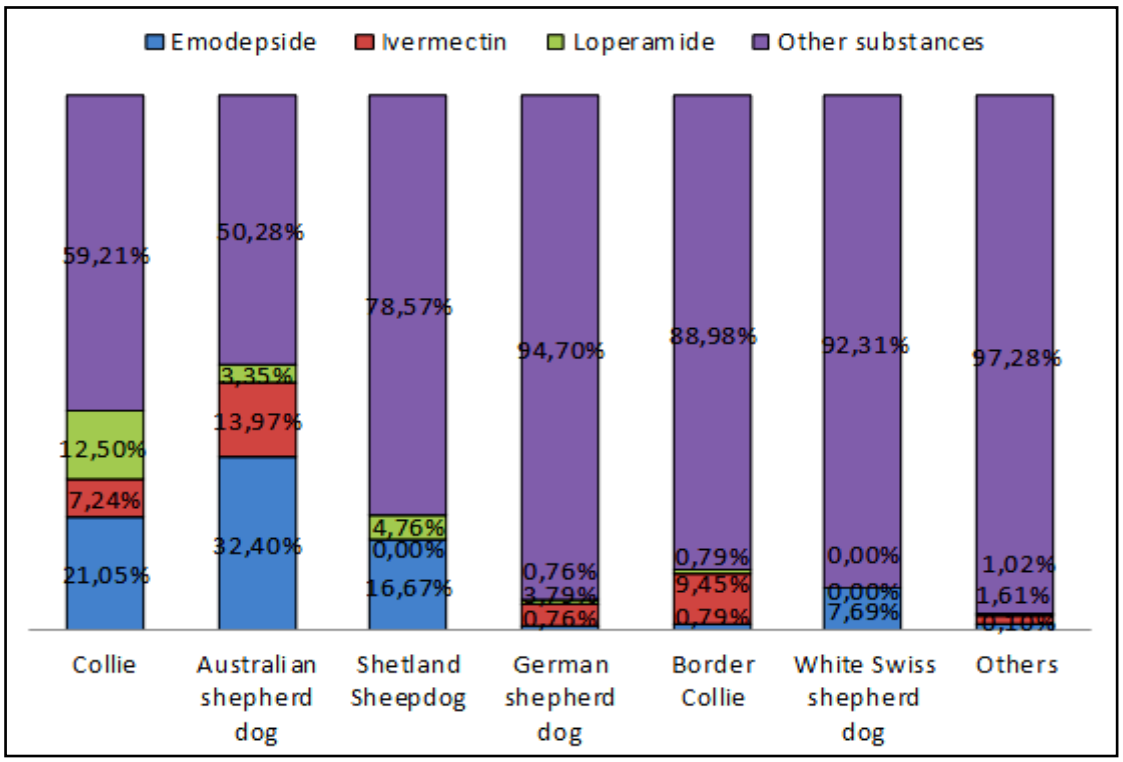
For Australian shepherd dogs 49,72% of ADRs are related with the molecules concerned. Special attention should be put in the use of emodepside, because this molecule represents 32,40% of the total ADR reported in this breed.

In Shetland sheepdog the same advice above is recommended (16, 67%).

German shepherd dogs and Border Collies have a low frequency of the MDR1m so, as can be seen, the molecules in analysis represent 5,30% and 11,02%, respectively, of the total ADRs in these breeds.

Concerning the White Swiss shepherd dogs, only ADRs related with the use of emodepside are reported, which represents 7,69 %.

Figure 21: Percentages of ADRs reported concerning the molecules ivermectin, loperamide and emodepside, considering the total of drug reports (n= 5529, CPVL, 2005-2011).



3.2 Intravenous lipid emulsions to manage avermectins intoxication

In addition to the records statements of suspected adverse effects and evaluation of the drug possible role, CPVL specialists are also required to advise veterinarians, by phone, on the necessary procedures, after intoxication or even in accordance with label use. For a better mutual assistance, CPVL is always looking for the best management of clinical cases, through a constant research in medical and scientific literature, to advise the finest and sometimes even an innovative treatment.

In 2011 CPVL recorded 201 notifications calls, in domestic carnivores concerning avermectins (poisonings, suspicion of adverse effects or asymptomatic ingestions). Regarding these problematic numbers and its consequences, the specialist of CPVL started to propose to veterinarians the use of ILE in cases where the prognosis seemed to be extremely reserved.

3.2.1 Objectives

As this treatment is innovative in Veterinary Medicine to manage avermectins intoxication the clinical cases, treated according CPVL indications, will be presented and studied.

3.2.2 Material and methods

The data was collected by two procedures. First, search into the Sentinel-vet database, to retrieve ADR with avermectins where therapy section contained “lipid”. Second, all the ADR for which lipid therapy protocol had been sent to vet by e-mail were controlled to see it had been performed. The advised protocol was the following: INTRALIPIDE 20%: IV administration (1, 5 mL/Kg during 1 minute), IV infusion of 0, 25 mL/ Kg/ min during 30 minutes and repeat the treatment, if necessary.

Since 1 January 2011, this treatment has been proposed, however its application was only possible in 6 cases, given the difficulty to obtain this type of medicine. Within these cases, only two breeds are not known to possess a strong proportion of the MDR1 mutant gene within their population.

3.2.3 Results

The follow table 17 presents a brief description of the clinical cases analised.

Table 17: Resume of clinical cases with ILE admistration (CPVL, 2011-2012).

	No/breed/ weigh/age	Exposure/Drug and type of administration.	Case description	Outcome.
1	2/ Australian shepherd dog.	**Accidental case with ivermectin:** two dogs were retained in a box where a horse was dewormed with paste containing this drug.	**2h:** One dog stars to present ataxia, incoordination, with decreased vision but normal pupillary reflexes, a slight mydriasis and anxiety. **8-10h:** the first dog assisted entered in coma and the other, previously asymptomatic, presented a posterior paresis and vomiting. **24h:** the second dog was completely recovered just with fluid therapy but the other one continued in coma. One infusion of **MEDIALIPIDES** was administered 0, 5 mL/kg/min during 30 minutes. The veterinarian did not find any improvement in animal condition and the animal died, probably by wrong route. No necropsy was performed.	1 recovered. 1 dead.
2	1/Border Collie /20 kg /3 years	**Accidental case with ivermectin:** dog ingested one ivermectin tablet that was destined to a donkey.	**Few hours later:** the dog started to have muscle tremors, ptyalism, decrease vision and mydriasis. However, the dog was conscious and responded to voice. The administration of ILE (unknown) led to the dog recovery. **2h:** later, the dog presented amaurosis that lasted 3 days.	1 recovered.

3	1/ Basset Hound / 34 kg.	**Accidental case with ivermectin:** received one ivermectin tablet destined to the treatment of equines.	**2-4h:** the dog was with ataxia, ptyalism, vomiting, amaurosis and then seizures. The patient received diazepam and one infusion of Ringer Lactate. The veterinary also administered atropine and the dog was anesthetized with medetomidine, ketamine and buprenorphine. **24h:** the dog was in a coma. After received **INTRALIPIDE 20%** (450 mL during 35 minutes) the animal improvement was excellent and the dog was discharged home. **72h:** One control visit to the veterinarian confirmed a complete recovery.	1 recovered.
4	1/ Australian shepherd dog / 30 kg/ 1 year.	**Accidental case with ivermectin:** the ingested some ivermectin during the dewormed of one horse.	**Few hours:** the dog had neurological disorders (no further details available). When the veterinarian did the clinical exam, the animal symptoms had progressed to a coma. The Veterinarian prescribed fluid therapy with Ringer Lactate + Glucose and administered activated carbon by intragastric tube. **12h:** already with symptoms, it was given an infusion of **INTRALIPIDE** (500mL). **24h:** the animal did not resist and died.	1 died.
5	1/ York shire/ 3 kg/ 2 years.	**Accidental case with ivermectin:** received 25 mg of ivermectin (2, 5 mL of a 1g/100mL solution) subcutaneous, administered by the owner.	The owner, after understanding his mistake, immediately went to a clinic and the patient did not present clinical symptoms when the veterinarian examined him. The product seemed completely absorbed at the administration site. The dog received activated carbon and fluid therapy with Ringer Lactate. **2h:** after accidental administration of ivermectin was given 6 mL of **INTRALIPIDE** to the patient and, later, a slow infusion with a rate of 1 drop every 15 seconds.	1 recovered.

			A new administration of INTRALIPIDE (1 hour infusion) was done to the dog **3h:** after the injection the dog presented few tremors and a slight ataxia. **5h:** the clinical signs completely regressed and, in the next morning, the dog was in good health, though, with mydriasis. **4 days:** the dog presented other troubles, like a persistent discomfort when he is exposed to light.	
6	1/ Border Collie/ 20 kg/ 5 years	**Suspected accidental case with eprinomectin:** The owner suspected that his animal had drunk milk from goats treated	The dog presented convulsions, opisthonos, muscles of abdomen tense and ptyalism. **8h:** After arrived in the clinic, the dog was given a symptomatic treatment, with diazepam, glycopyrrolate, Ringer Lactate infusion, butylscopolamine/dipyrone, hepatic protectors and corticoids. After a brief clinical recovery phase, the animal started to relapse and the veterinary administered doxapram and tiletamine/zolazepam to anesthetize. Later, the dog received an infusion of **OLICLINOMEL** (500mL). **48h:** after the admission in the clinic the animal died.	1 died.

3.2.4 Discussion

The analysis of these 6 cases, which used ILE as treatment, showed that 4 animals recovered and 3 died. It is obvious that the number of cases is not enough to an accurate analysis, although, with these cases and comparing to the present literature it is possible to identify some trends.

The first point to discuss is the veterinarian difficulty to obtain these medicines. In France, the most important products in the market are: INTRALIPIDE, IVELIP, MEDIALIPIDE and OLICLINOMEL. The INTRALIPIDE seems to be the product with more potential to treat the intoxication cases. However, it is also the less well absorbed and tolerated in parenteral nutrition. MEDIALIPIDE has also soybean oil while OLICLINOMEL is an emulsion of olive oil. In the 6 reported cases to CPVL, the MEDIALIPIDE and OLICLINOMEL were once used and INTRALIPIDE was applied tree times. In one case it was not described the used product. Taking in account the received cases, INTRALIPIDE was the easiest product to obtain. Possibly because it is recommended in protocols for intoxication treatment by local anesthetics in humans (LIPIDRESCUE) and it is the one with more data available.

Table 18: Lipid products used as treatment and their composition (adapted from: Velde V. et al, 1996).

Product name	Composition
INTRALIPIDE	Soybean oil 10 or 20%
IVELIP	Soybean oil 10 or 20%
MEDIALIPIDE	Soybean oil 10% and Medium-chain triglycerides 10%
OLICLINOMEL	Olive oil 16% and Soybean oil 4%

Case 1: MEDIALIPIDE was only used in this case. The patient was already in coma for 24 hours before the product administration. The ingestion of ivermectin was just a suspicious and no quantification of the ingested dose was possible. The symptoms started quickly and developed to coma. One animal died after MEDIALIPIDE administration, however, any adverse effects was noticed.

Case 2: this case consists of the administration of an unknown lipid emulsion, which allowed a rapid recovery of the Border collie, which ingested a high dose of ivermectin. Despite the absence of data related to the dose and the name of the product used to treat, the unexpected patient recovery, within 1 hour after administration, suggests an efficacy of the treatment performed according CPVL recommendations.

Case 3: After 24 hours of coma the animal dramatically improved its disorder with a total symptoms remission and just in a few hours after INTRALIPIDE administration.

Case 4: The mortal case followed INTRALIPIDE administration according to the protocol proposed by CPVL. The animal was, however, already in coma and Australian Shepherd dogs are known for possessing a great proportion of MDR1 mutants and the quantity of ivermectin ingested is unknown.

Case 5: The dog was treated in a preventive way, presented the signs later as ataxia, transient tremors for 2 hours and mydriasis (persisting several days). However, it should be taken into account that this dog had received approximately 8mg/kg of ivermectin, which makes 40 times one therapeutic dose. In this case seems obvious the favourable influence of INTRALIPIDE injection against ivermectin intoxication and no undesirable effects were reported.

Case 6: In the other case, which used OLICLINOMEL, the patient presented convulsions after a possible contact with eprinomectin. Although, this product does not belong to the group of the most effectives for intoxication treatments, this case contains a numerous of uncertainties, namely, the troubles origins and the possible ingested dose. These factors limit the capacity to judge the inefficacy obtained with the ILE. Additionally, the implicated breed was a Border collie, with a strong predominance of the gene MDR1m.

4 Conclusion

Pharmacovigilance and pharmacogenetics are two branches of science that have everything to gain if they work together. Pharmacogenetics can give a more mechanistic and scientific assessment to the ADRs and pharmacovigilance can give their data, continuously collected, that may contain indications of genetic particularities between species and even among the same breed. One example of this useful collaboration is the Collies case.

It took more than 20 years to understand and identify the cause of higher sensitivity of most individuals belonging to the Collies breed. Now, it is known that happens due to the MDR1 gene mutation (4-bp deletion), which origin non-functional Pgps and, consequently, affects its role on xenobiotics efflux leading to intoxications with some common used molecules. The mutation in case is present in 11 canine breeds and the genetic screening tests seem to be the best approach to this genetic disease - "white feet, test to see if you can treat". However, many substrates of Pgp have not been yet identified, as well, as some dangerous interactions during simultaneous use.

Through the collection and analysis of data from the Sentinel-Vet software, it was possible to find out some important deductions. During the time between 2005 and 2011, ivermectin was the 15th more reported molecule to CPVL, in dogs. Emodepside and Loperamide are, respectively, in 17th and 56th place. After administration of the substances in cause, the most common symptoms reported were related with the nervous system, and this agrees with what was expected.

The low results of ADRs due the use of ivermectin in the sensitive breeds, especially in Collies, may be owed to the conscience of veterinarians and owners because the SPC of medicines containing avermectins had this warning since 2001. However, when analyzed data with the serious ADRs reported, these groups represent 52% of the total. More, statistical analyses confirmed accidental cases as the principal cause of contact with this product and the owners are the main responsible for the administration.

Relatively to loperamide the numbers are impressive. 73% of ADRs are represented by breeds knowing to be carriers of the MDR1m, once administered by the owners.

Emodepside is a comparatively new antiparasitic and the statistical analysis shows a great fraction of breeds known to be carriers of MDR1 mutant gene, they represent 69% of the total reports.

Through the general analysis we are able to prove that our data are realistic. The proportion of ADRs reaction reports containing only breeds without the mutant allele showed that

ivermectin, loperamide and emodepside represents a very low fraction, comparative with the individual breeds, known to be carriers.

The number of cases recorded after ILE used, in response to avermectin intoxication, is not enough to make a valid statistical study. It is also not conceivable to evaluate the therapeutic efficacy or compare the effectiveness of the different emulsions available in the French market. It remains, however, that these results involved fast remissions of the acute symptoms, specialty with INTRALIPIDE 20%, even in dogs belonging to the affected breeds. In this small case samples no adverse effects to the treatment were mentioned. Subsequently, and adding the high numbers of avermectin intoxications reports to CPVL, the study must go on to allow a better knowledge of effectiveness and optimal application conditions.

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Annex 1. DGAV – Internship report

Relatório relativo ao estágio efetuado na Direção Geral de Alimentação e Veterinária.

- **Introdução.**

O mestrado integrado em Medicina Veterinária pela Faculdade de Medicina Veterinária da Universidade Técnica de Lisboa apresenta no seu plano curricular um estágio final, que corresponde ao 6º ano do MIMV. Este estágio tem uma componente prática que deverá corresponder a 500 horas, sendo que no final o estágio em si corresponde a 30 ECTs. No nosso caso o estágio oficial, de 4 meses, foi realizado ao abrigo do programa Erasmus, na *École Nationale Vétérinaire de Lyon,* mais concretamente no *Centre de Pharmacovigilance Vétérinaire de Lyon*, (CPVL). O tema da dissertação correspondente terá por base as atividades realizadas nesse estágio. Contudo, consideramos igualmente importante o contacto com a realidade nacional e com uma parte que aborde mais a componente legislativa/regulamentar dos medicamentos de uso veterinário. Assim sendo, e com o objetivo de complementar a formação, foi solicitado o estágio na atual Direção Geral de Alimentação e Veterinária (DGAV), na respetiva Direção de Serviços de Medicamentos e Produtos de Uso Veterinário. Durante aproximadamente dois meses o estágio decorreu, então, no gabinete de farmacovigilância veterinária com supervisão do Dr. Henrique Ramos da Costa.

- **Desenvolvimento**

O Sistema Nacional de Farmacovigilância Veterinária (SNFV) tem como objetivo a recolha de informações relacionadas com eventos adversos e involuntários, nos animais ou, eventualmente, no homem, quando expostos a produtos utilizados no domínio da produção, saúde e bem-estar animal.

No contexto da Farmacovigilância **evento adverso** (EA) é qualquer reação nociva e involuntária a um medicamento que ocorra com doses geralmente utilizadas no animal na profilaxia, no diagnóstico ou tratamento de doenças, ou na recuperação, na correção ou na modificação de funções fisiológicas. Pode ser considerado não-grave ou grave.

Apesar de serem submetidos a rigorosos ensaios nas fases prévias à autorização de introdução no mercado (AIM), que decorrem em ambiente laboratorial e em determinadas amostras populacionais, estes podem não ser suficientes para garantir a segurança efetiva dos medicamentos veterinários incluindo os imunológicos, os produtos de uso veterinário e os biocidas de uso veterinário. Isto porque quando são colocados no mercado as situações de aplicação são infinitamente vastas. Por exemplo, o medicamento poderá ser aplicado não só na população alvo, que por norma apresenta diferentes situações patológicas, mas poderá também ser administrado no uso extra-indicações, nomeadamente o decorrente da aplicação da cascata. Assim sendo, após a obtenção da AIM, todos os medicamentos utilizados em saúde animal têm que continuar a ser vigiados, para continuarem a cumprir as exigências de qualidade, eficácia e segurança e oferecer um balanço benefício/risco aceitável.

No sistema português qualquer pessoa pode notificar uma reação adversa (médicos veterinários, profissionais de saúde ou quaisquer outras fontes), sendo esta posteriormente imputada de acordo com o sistema ABON.

Avaliação da causalidade:

- Categoria "A" - PROVÁVEL;
- Categoria "B" - POSSÍVEL;
- Categoria "O" - NÃO CLASSIFICÁVEL
- O1 - INCONCLUSIVO (casos em que outros fatores não permitem uma conclusão, mas em que a associação ao produto não pode ser descartada);
- e Categoria "N" - NÃO RELACIONADO com o medicamento.

As ações regulamentares tomadas variam em função do resultado posterior desta análise e podem contemplar alterações ao Resumo das Características do Medicamento (RCM) como: a adição de advertências ou contraindicações; a alteração da (s) via (s) de administração; ou a eventual recolha do produto (ou lote); e mesmo a suspensão ou revogação da AIM.

O Sistema Nacional de Farmacovigilância Veterinária encontra-se legislado no Decreto-Lei n.º 148/2008, de 29 de julho – Capítulo XI, art.^{os} 108.º a 112.º, modificado pelo Decreto-Lei n.º 314/2009, de 28 de outubro. A legislação dos medicamentos veterinários estabelece um conjunto de obrigações e responsabilidades, quer para o responsável pela AIM, quer para a autoridade competente (DGAV). Neste conjunto de obrigações e responsabilidades, está instituído que o detentor da AIM deve dispor de um diretor técnico veterinário que deve conceber e gerir um sistema de farmacovigilância veterinária, que garanta a recolha de toda a

informação relativa a todas as suspeitas de reações adversas comunicadas a qualquer pessoa que se encontre ao serviço da empresa, e que a mesma seja avaliada e coligida de modo a estar disponível em, pelo menos, um lugar determinado na Comunidade Europeia.

No Volume 9B das "Regras que Regem os Medicamentos Veterinários na União Europeia" *"Guidelines on Pharmacovigilance for Medicinal Products for Veterinary Use"* estão descritos todos os aspetos que os detentores da AIM devem observar e executar para terem um sistema de farmacovigilância funcional.

O SNFV abrange igualmente outros aspetos da vigilância pós-comercialização, tais como:

- Suspeita de reação adversa associada ao uso não contemplado na rotulagem;
- Falhas da eficácia prevista.
- Investigação da validade do intervalo de segurança (para os casos de deteção de resíduos apesar de a dose e o intervalo de segurança terem sido cumpridos).
- e Possíveis problemas ambientais.

Neste âmbito e no decorrer do estágio foram realizadas as seguintes ações:

- Elaboração da avaliação de relatórios periódicos de segurança (RPS), quer de medicamentos veterinários farmacológicos, quer de medicamentos veterinários imunológicos que têm como objetivo a avaliação da relação benefício-risco, essencialmente focalizada na avaliação do risco. Nesta área de atuação várias questões se nos colocaram. Isto porque se coloca a possibilidade da diminuição da periodicidade de emissão dos mesmos, ou de serem apenas produzidos após determinação por parte das autoridades. De um determinado modo concordamos com este fato, pois a existência e o funcionamento cada vez mais eficaz do "Eudravigilance Veterinary", com um incremento nomeadamente da "Signal Detection", será suficiente. Outro ponto a salientar será a diferença entre o formato dos RPS submetidos, pois consideramos que deveria existir uma maior uniformidade entre eles. Por vezes torna-se difícil a avaliação devido aos diferentes padrões usados pelas diferentes empresas. Imputação de notificações de eventos adversos através do sistema ABON. Foi bastante interessante pois tivemos oportunidade de fazer a imputabilidade de diferentes eventos adversos que ocorreram no período anterior a este estágio. Neste aspeto foi possível constatar que, em relação à imputação, por vezes a indústria e as autoridades têm pontos de vista algo diferentes. Para esta situação podem contribuir os diferentes fatores de imputação de um Evento Adverso (EA): a conexão associativa, a

explicação farmacológica e conhecimento prévio do medicamento, a presença de fenómenos caracterís*ticos clínicos ou patológicos, a exclusão de ou*tras causas, a fidedignidade dos dados, a relação temporal (incluindo paragem e recomeço da terapêutica), a localização anatómica, e a medição quantitativa do grau de contribuição de um medicamento para o desenvolvimento de uma reação (relação dose-efeito).

- Introdução e utilização do sistema "Eudravigilance Veterinary". O portal EudraVigilance está em funcionamento na European Medicines Agency (EMA) desde Dezembro de 2001 para o setor dos medicamentos humanos e desde 2005 para o setor dos medicamentos veterinários e permite uma dinamização e melhoria da transmissão, por via eletrónica, de relatórios de segurança individuais. Este sistema envolve todos os medicamentos autorizados e comercializados na UE e a informação é disponibilizada a todas as entidades reguladoras e aos titulares de AIM a referente a casos ocorridos com os respetivos medicamentos. Este programa tem uma opção para a realização de "testes", permitindo uma melhor compreensão e aplicação do sistema.

Na parte final do estágio pudemos ainda contactar com os aspetos e atividades relativas ao licenciamento da atividade de distribuição de medicamentos veterinários farmacológicos e imunológicos (distribuição por grosso e venda a retalho) e de alimentos medicamentosos (fabrico e distribuição por grosso). Procedemos assim ao acompanhamento da Dr.ª Maria da Luz Grencho em vistorias a diversas entidades no âmbito dos Diplomas em vigor. Em todas elas foi possível seguir uma "guia-chave", que serviu de base para a realização das vistorias e compreensão da conclusão final alcançada. Foram acompanhadas as seguintes vistorias: venda a retalho de medicamentos veterinários- Clínica veterinária "115 animal" localizada em Olhão; distribuição por grosso de medicamentos veterinários - filial da empresa "Medinfar" localizada na zona do Algarve; fabrico e distribuição por grosso de alimentos medicamentosos – fábrica da "Promor" localizada na zona industrial de Leiria.

Conclusão:

Os medicamentos e produtos de uso veterinário podem representar um risco não somente para a saúde e bem-estar animal, mas também para os seres humanos que de maneira direta ou indireta se expõem a estas substâncias. Médicos veterinários, proprietários entre outros, além dos consumidores de alimentos de origem animal, estão então expostos aos potenciais efeitos adversos destes medicamentos/produtos de uso veterinário, representando então a Farmacovigilância Veterinária um campo de atuação para a prática da Saúde Pública.

Relativamente ao estágio, é nossa opinião que todos os objetivos traçados para este foram alcançados e as perspetivas inclusivamente ultrapassadas.

Constituiu uma experiência que permitiu consolidar os conhecimentos já adquiridos no primeiro estágio realizado no CPVL. O sistema francês de farmacovigilância é único na Europa, funcionando de um modo muito particular. No CPVL estabelece-se uma relação de "mutualismo", com as pessoas que apelam ao centro, existindo uma troca de "saberes". O médico veterinário/dono fica a saber quais os procedimentos mais adequados a aplicar na situação de emergência e em troca o CPVL recolhe a notificação da RA. Este sistema gira à volta deste centro e não ao nível das autoridades como em Portugal. Os relatórios são arquivados num programa, o Sentinel-Vet, e as notificações graves devem ser reportadas num prazo máximo de 15 dias. As não-graves apenas devem ser declaradas de 3 em 3 meses à *Agence Nationale du Médicament Vétérinaire* (ANMV), que posteriormente recomenda às autoridades as medidas a serem tomadas para minimizar os riscos de efeitos adversos.

Podemos sem dúvida concluir igualmente que a possibilidade de contactar com todos estes excelentes profissionais e sentir que contribuímos um pouco, igualmente, para esta instituição, foi bastante gratificante quer a nível profissional, quer a nível pessoal.

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