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Evaluation of the ABCB1 genotype and risk factors in dogs affected by refractory idiopathic epilepsy

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ABSTRACT

Despite the advances in the treatment of Idiopathic Epilepsy, still a relevant percentage of dogs don't achieve an adequate seizure control despite the association of different anti-epileptic drugs (AEDs) at appropriate dosage. This condition, know as Refractory Epilepsy, is considered one of the most frustrating condition for pets, owners and neurologists. In human and veterinary medicine, great effort is spent in trying to elucidate the mechanisms underlying responsiveness and refractoriness to AEDs treatment.

Recently, attention has been focused on the attempt to identify risk factors to predict the outcome of the disease and to understand the exact pathophysiological mechanisms of RIE. A key role seems to have P-glycoprotein encoded by the ABCB1 gene. It has been supposed that an overexpression of these efflux transporters, due to an ABCB1 mutation, may inhibit AED penetration in epileptic foci resulting in a reduced efficacy of antiepileptic treatment. A similar mechanism was hypothesized also in veterinary medicine, indeed a single nucleotide variation (SNV) of *ABCB1* gene (c.-6-180T>G) has been associated with phenobarbital-resistance in a population of idiopathic epileptic Border collie.

In the present study, data from a population of Refractory Idiopathic Epileptic dogs (RIE-dogs) were statistically compared with a control group of AED-responsive dogs to identify clinical risk factors associated with RIE and the frequency of the ABCB1 c.-6-180T>G SNV were assessed in this multi-breed population affected by RIE. The present study confirmed that clinical risk factors for RIE include the early onset of seizures and the experience of cluster seizure and identified a higher risk to develop RIE in the Cane Corso and in the Border Collie breed. Furthermore, the study confirmed the presence of the c.-6-180T>G polymorphism in several breeds and failed to identify any association with RIE.

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SUMMARY

Despite the advances in the treatment of Idiopathic Epilepsy, still a relevant percentage of dogs don't achieve an adequate seizure control with the association of different anti-epileptic drugs (AEDs) at appropriate dosage (Trepanier et al., 1998; Schwartz-Porsche et al., 1985; Podell and Fenner, 1993). This condition, know as Refractory Epilepsy is considered one of the most frustrating condition for pets, owners and neurologists (Muñana, 2013). In human and veterinary medicine, great effort is spent in trying to elucidate the mechanisms underlying responsiveness and refractoriness to AEDs treatment (Remy and Beck, 2006; Schmidt and Löscher, 2005; Muñana et al., 2012; Alves et al., 2011).

Recently attention has been focused on the attempt to identify risk factors to predict the outcome of the disease and to understand the exact pathophysiological mechanisms of RIE. A key role seems to have the P-glycoprotein encoded by the ABCB1 gene (Kwan and Brodie, 2005). It has been supposed that an overexpression of these efflux transporters, due to an ABCB1 mutation, may inhibit AED penetration in epileptic foci, resulting in a reduced efficacy of antiepileptic treatment (West and Mealey, 2007). A similar mechanism was hypothesized also in veterinary medicine, indeed, a single nucleotide variation (SNV) of *ABCB1* gene (c.-6-180T>G) has been associated with phenobarbital-resistance in a population of idiopathic epileptic Border collie (Alves et al., 2011).

The aims of this multicentric study consisted in: providing a detailed description of the clinical presentation aimed to identify possible clinical risk factors and assessing the frequency of the *ABCB1* c.-6-180T>G SNV in a multi-breed population of dogs affected by RIE

Three parts compose this thesis. According to recent literature, the first part describes the seizure pathophysiology, the classification system of epilepsy and the clinical, diagnostic and therapeutic approach to the epileptic dog (**Chapter I**).

The second part focuses on the definition of refractory epilepsy, on clinical and genetic factors associated with a poor drug-response and on main hypothesis of the pathogenetic drug-resistance mechanisms (**Chapter II**).

In the **Chapter III** (the experimental part) data from a population of Refractory Idiopathic Epileptic dogs (RIE-dogs) were statistically compared with a control group of AED-responsive dogs to identify clinical risk factors associated with RIE. Furthermore, the frequency of the ABCB1 c.-6-180T>G SNV was assessed, as possible genetic risk factor in this multi-breed population affected by RIE.

CHAPTER I

CANINE EPILEPSY

1.1 HISTORY OF EPILEPSY

The word *epilepsy* derives from the Greek word *epilambanein,* which means “to be taken”.
This definition perfectly reflects the feutures of a seizure; it suddenly appears and spontaneously stops. For these characteristics of unpredictability and inexplicability, in ancient times, epilepsy was called the “sacred disease”, and humans suffering from epilepsy have been thought to be insane or possessed by demons.
The Greek philosopher Hippocrates (460-377 BC) was the first person to think that epilepsy starts in the brain. Afterward, Galen viewed the epileptic seizure as a symptom of intracranial dysfunction or systemic disease, caused by an accumulation of mucous in the arterial system.
During the Middle Ages**,** the bilief that people with epilepsy were under a demonic or other spiritual possession came back. Therefore, the treatment of epilepsy included practices as exorcisms and bloodletting.
In the late 19th century, the physician John Hughlings Jackson came to the conclusion that epileptic seizures were due to an abnormality of "excessive neuronal discharge" in the cortical gray matter. This finding led to a series of studies that investigated the origin of focal cortical changes as a cause of seizures.
At the beginning of the XX century, there was a futher evolution with the introduction of new therapeutic approaches based on Jackson's theories. In 1912, Alfred Hauptmann discovered the anticonvulsant activity of the phenobarbital.
Some years later, in 1924, Hans Berger, a German psychiatric, succeeded in recording the first human elettroencephalogram that allowed a better understanding of the epileptogenesis mechanisms.

1.2 SEIZURE PATHOPHYSIOLOGY

The epileptic seizure has been defined as a transient occurrence of signs due to abnormal, excessive or synchronous neuronal activity in the brain (Fisher et al., 2005). The main feature of all epileptic syndromes is a persistent increase of neuronal excitability (Platt, 2014a). The epiletic activity results from an imbalance between excitatory and inhibitory mechanisms due to a modification in the normal neuronal circuit (March, 1998).

In order to properly understand the pathophysiology of seizures is of paramount importance having a good knowledge of the normal neuronal properties.

The neuron is a polarized cell. It shows an unequal distribution of sodium ions (Na+) and potassium ions (K+) across the membrane. This different distribution produces a resting membrane potential (RMP) of approximatelly -70 millivolts. The RMP is maintained by the selective permeability of the plasma membrane to some ions and by an energy dependent sodium-potassium pump. The pump, extruding actively the sodium out of the cell and pumping the potassium (in a ratio of 3:2) inside keeps the internal of the cell more negative (Klein and Cunningham, 2013a). After the release of a neurotransmitter from a presynaptic axon terminal, a local reversal in membrane potential occurs. According to the function of the neurotransimitter released, a the local change in the permeability of the cell membrane results in an influx of positive or negative charged ions and, respectively, in a state of depolarization or hyperpolarization. The depolarization is the consequence of an excitatory postsynaptic potential (EPSP) and the hyperpolarization of an inhibitory postsynaptic potential (IPSP) [(March, 1998)]. EPSP and IPSP are both local events.

When the simultaneous ("*Spatial summation*") and/or the rapid succession ("*Temporal summation*") release of depolarizing neurotransmitters reach a certain threshold (-55mV) at the axon hillock, an *Action potential* is generated (King, 1999). The action potential is the result of the sequential opening of voltage-gated ion channels in the cell membrane, first to the sodium and then to the potassium. The dramatic influx of Na+ ions that accompanies action potential depolarization of the initial segment's membrane, results in the passive spread of positive charges toward the adjacent resting segment of the axonal membrane. This positive charge migration allows the transmission of the nervous impulse. (Klein and Cunningham, 2013b).

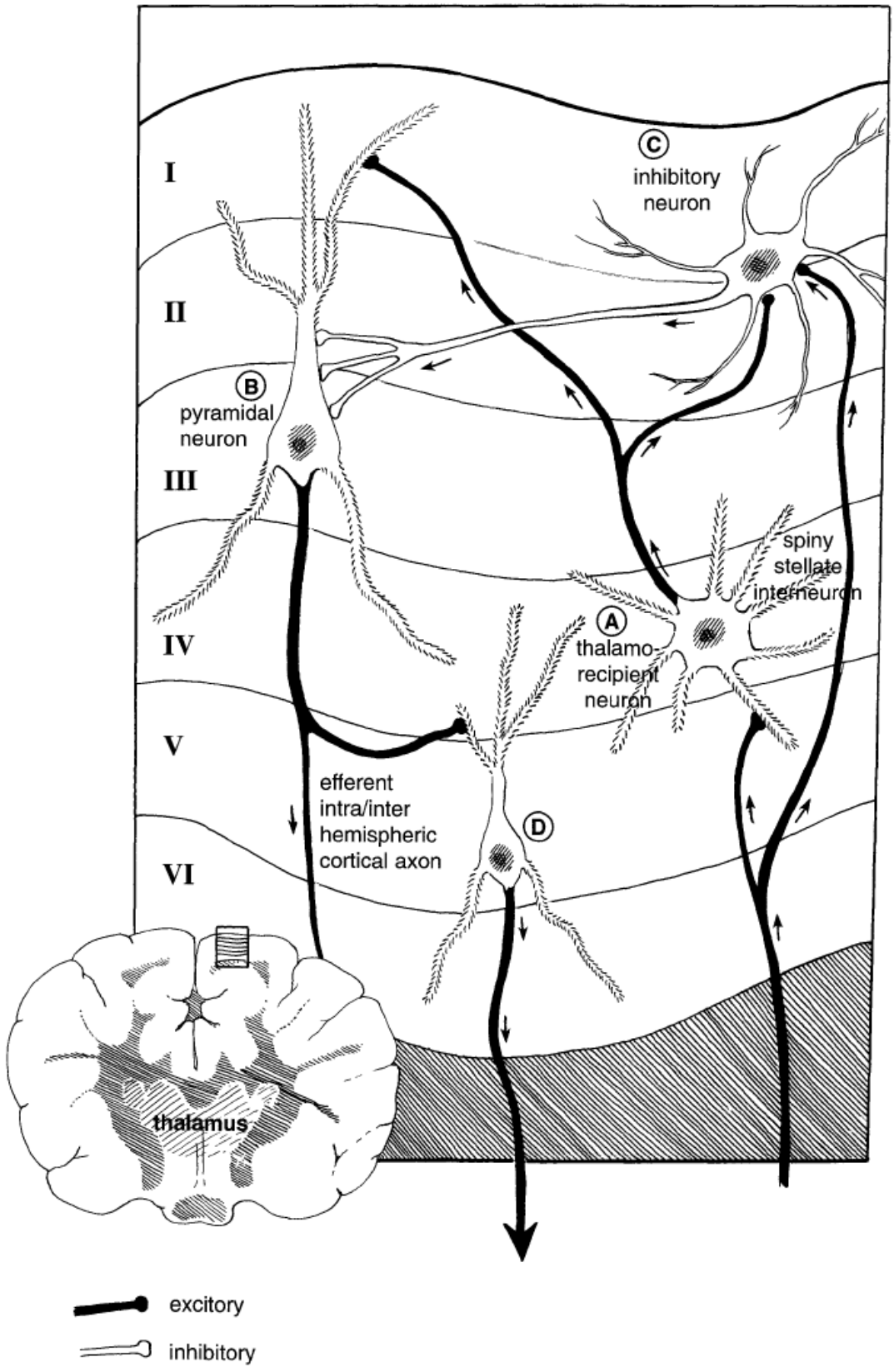
Conversely, spatial or temporal summation of IPSPs results in hyperpolarization of the cell membrane and the maintenance of a resting state.

The principal inihibitory neurotransmitter in the cerebral cortex is the γ-Aminobutyric acid (GABA), acting on two types of GABA receptors: $GABA_A$ e $GABA_B$ receptors. $GABA_A$ receptors

are ligand-gated ion channels that hyperpolarize the neuron by increasing inward chloride conductance. $GABA_B$ receptors hyperpolarize the neuron by increasing potassium conductance and decreasing calcium entry (Treiman, 2001). The predominant excitatory neurotransmitter is the glutamate, which acts on several receptors: the α-*amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAr)*, crucial for fast excitatory neurotrasmission, the *N-methyl-D-aspartate receptor (NMDAr),* mediating the slow post synaptic excitatory potentials and *Kainate receptor (KAr)* which role has not yet been entirely understood (Barker-Haliski and White, 2015).

An imbalance between excitatory and inhibitory neurotrasmission underlies the mechanism of the epileptic seizures. The seizures develop when the balance shifts towards excessive excitation. In the epileptic patient, the excessive excitation and/or the lack of inhibition lead to the paroxysmal depolarization of neurons without normal regulatory feedback mechanisms. This is particularly true in certain brain areas more sensitive to depolarization (trigger or epileptic foci). The result is a *paroxysmal depolarization shift* of the neuronal aggregate. In response to this sudden change in brain activity, the local surrounding inhibitory zones try to prevent the spread of this epileptogenic activity. When the inhibition is unsuccessful, other neuronal aggregates are excited through thalamocortical recruitment. The recruitment of a critical number of areas with synchronized depolarization may lead to a seizure (Podell, 2013).

The sequence of events converting a normal neuronal network in a hyperexcitable network is called "*epileptogenesis*" (Patterson, 2013). These can be induced by several causes, such as brain lesions or genetic factors. Brain lesions (e.g. trauma, hypoxia, and ischemia) can produce epileptic foci through the synaptic reorganization of the neuronal circuits. Indeed, after a brain injury, excitatory axons may sprout new collateral axons producing aberrant connections and breaking down the inhibitory circuits (March, 1998). Among the genetic epilepsies in human medicine, one of the possible causes of epileptogenesis is the dysfunction of mutated voltage- or ligand-gated ion channels ("channelopathies") or mutation of the neurotransmitters (Avanzini et al., 2007).



Schematic representation of the neuronal circuitry in the cerebrum responsible for feed-forward excitation and feed-forward inhibition. An imbalance in the levels of excitation and inhibition can lead to seizure discharges (from March, 1998).

1.3 CLASSIFICATION OF SEIZURES

In veterinary medicine, the terms used to classify seizure and epilepsy have been debated for a long time. In the last three decades, several classifications have been proposed, sometimes reflecting the authors' preferences. For this reason, they lacked uniformity in the definitions (Schwartz-Porsche, 1994; Berendt and Gram, 1999; Licht et al., 2002; Berendt, 2004; Podell, 2004; Thomas, 2010). With the aim to uniform the terminology concerning seizures and epilepsy, the veterinary classification of seizures has progressively tried to be more compliant with the guidelines proposed by ILAE (International Legue Against Epilepsy) for the human medicine.

ILAE is a renowned association of "*physicians and other health professionals working towards a world where no persons' life is limited by epilepsy*" (ILAE homepage www.ilae.org). ILAE provides a universally recognized vocabulary to facilitate the communication among the scientific community, allowing the comparison between clinical and basic research on epilepsy (Engel, 2001). The first classification of the Commission on Classification and Terminology of the ILAE was published in 1969, updated in 1981 for seizures (Commission, 1981) and in 1989 for epilepsies (Commission, 1989). Afterwards, a new consensus document was produced approximately every 5 years, on the basis of the growing knowledge on the disease.

Despite the veterinary terminology is largely compliant with ILAE classification, many significant differences between humans and animals have been considered to avoid the passive acquisition of terms unfit to properly define seizures and epilepsy in animals. Unlike humans, in pets the recognition of seizure occurrence and its clinical manifestations is mainly dependent on the owner's observation; electroencephalographic (EEG) data are usually not available (De Risio, 2014a), and, sometimes, the owners because of financial reasons refuse diagnostic investigation. In 2014 the International Veterinary Epilepsy Task Force (IVETF) was founded in order to produce consensus statements on veterinary epilepsy, (Volk, 2015). IVETF has proposed a classification system reflecting the human ILAE guidelines, taking into consideration the well-accepted terminology (Berendet et al., 2015).

According to the recent IVETF publications, ***epileptic seizure*** is **"**a transient occurrence of signs due to abnormal excessive or synchronous neuronal activity in the brain**"** (Berendet et al., 2015; Fischer et al., 2005, De Risio, 2014a) and ***epilepsy*** "a disease of the brain characterized by an enduring predisposition to generate epileptic seizures. This definition is usually practically applied as the occurrence of two or more unprovoked epileptic seizures at least 24 h apart" (Fisher et al., 2014; Berendt et al., 2015).

According to this definition, not all the seizures are associated with epilepsy. Seizures occurring as a natural response of the normal brain to a transient disturbance, such as a metabolic or toxic insult, and that disappear when the cause is solved are called ***reactive seizure.*** In this case, the patient does not suffer from epilepsy (De Risio, 2104a).
The IVETF system of classification is based on seizure frequncy, etiology and semiology (Berendt et al., 2015).

1.3.1 CLASSIFICATION OF SEIZURES BASED ON FREQUENCY

Epileptic seizures are classified as ***single seizure,*** if is a seizure occurs only once in a 24-hours period, and c***luster seizures,*** if two or more seizures occur within 24 hours with full recovery of consciousness between seizures. Furthermore, ***Status epilepticus*** is a condition characterized by continuous seizure activity for five or more minutes, or two or more discrete seizures within 24 hours without full recovery of consciousness between seizures (Berendt et al., 2015).

1.3.2 CLASSIFICATION OF EPILEPSY BASED ON AETIOLOGY

Recently, ILAE has adopted a new classification defining three categories: genetic epilepsy, structural/metabolic epilepsy and epilepsy of unknow origin (Berg et al., 2010).
In veterinary medicine, the earlier terminology classified epilepsy based on etiology, distinguishing: ***idiopathic*** (or primary), ***symptomatic*** (or secondary) and ***probably symptomatic*** (or criptogenic) epilepsy (Gandini, 2015a).
According to the new ILAE classification, in 2015 IVETF proposed to consider: ***idiopathic epilepsy***, ***structural epilepsy*** and ***epilepsy of unknow causes*** (Berendt et al., 2015).

Idiopathic epilepsy refers to recurrent seizures with no underlying cause other than a strongly

suspected or confirmed genetic basis.

The term idiopathic does not imply a disorder of unknown cause, referring to a recognized clinical syndrome with typical clinical features. These include the age at onset (between 6 months and 6 years), normal interictal behaviour, normal physical and neurological examination, and the exclusion of metabolic, toxic and structural cerebral disorders after proper diagnostic investigations (Platt and De Risio, 2014).

Idiopathic epilepsy includes three subcategories:

- ✓ ***genetic*** –when a causative gene for epilepsy has been identified/confirmed genetic background
- ✓ ***suspected genetic*** – when a genetic influence is strongly suspected but the causative gene/s has/ve not been discovered. Suspected genetic epilepsy is the term used in presence of a high breed prevalence (>2%), positive pedigree analysis and/or familial accumulation of epileptic individuals.
- ✓ epilepsy of ***unknown cause*** - when the nature of the underlying cause is unknown and there is lack of evidence of structural epilepsy (Berendet et al., 2015).

Structural epilepsy is characterized by epileptic seizures provoked by intracranial/cerebral pathology including vascular, inflammatory/infectious, traumatic, anomalous/developmental, neoplastic and degenerative diseases, confirmed by diagnostic imaging, cerebrospinal fluid examination, DNA testing or post mortem findings (Berendet et al., 2015).

Unkown causes epilepsy refers to recurrent seizures caused by a strongly suspected underlying brain disease, which cannot be identified despite adequate investigation (De Risio, 2014a; Mariani, 2013; Podell, 2013).

1.3.3 CLASSIFICATION OF EPILEPSY BASED ON SEMIOLOGY

The classification of epilepsy based on semiology distinguishes seizures in *focal-onset seizure* and

generalized-onset seizure.

Focal-onset epileptic seizures (previously named as partial seizure) are characterized by lateralized and/or regional signs due to an abnormal electrical activity arising in a localized group of neurons or network within one hemisphere. The old nomenclature classified focal seizures in simple or complex focal seizures based on the consciousness impairment. Unfortunately, during a focal seizure, the animal can be awake but disoriented and/or not able to recognize the owner nor to respond to commands. For these reasons, the state of consciousness cannot be assessed objectively. Therefore, the new terminology avoids subclassification of focal seizures based on consciousness. Rather, it is now suggested to describe the clinical expression of the seizure.

The clinical expression of a focal-onset epileptic seizure reflects the functions of the part of the brain involved. For this reason, focal-onset epileptic seizures can present tehmselves as:

- **Focal Motor seizure,** consisting of abnormal movements of a body part, such as facial twitches, repeated jerking head movements, rhythmic blinking, twitching of facial musculature or repeated rhythmic jerks of one extremity (Berendet et al., 2015). Focal-onset motor seizures are presumed to arise from a seizure focus near a primary motor area in the frontal cortex contralateral to the observed involuntary motor activity (De Risio, 2014a).
- **Focal Autonomic seizure** is characterized by parasympathetic and epigastric components as dilated pupils, hypersalivation or vomiting.
- **Focal Behavioural seizure** in humans can present psychic and/or sensory seizure phenomena, while in animals it results in a short lasting episodic change in behaviour such as anxiousness, restlessness, unexplainable fear reactions or abnormal attention seeking/'clinging' to the owner (Berendet et al., 2015).

Any type of ***focal-onset*** seizure can ***evolve into a generalized*** seizures. They are charaterized by a focal ictus consistent with the location of the seizure focus, that in seconds to minutes spreads, involving both cerebral hemispheres and resulting in bilaterally symmetrical motor disturbances (usually tonic-clonic), autonomicdysfunction and (commonly) altered consciousness. The focal onset may occur so rapidly that could be undetected and the seizure is misclassified as a generalized-onset seizure (Berendet et al., 2015; De Risio, 2014a).

In **generalized-onset epileptic seizures,** the clinical expression supports the immediate and simultaneous involvement of both cerebral emispheres. The motor manifestations are bilateral and consciousness alteration may frequently occur (Thomas, 2010). The main types of generalized

seizures are ***tonic, clonic*** or ***tonic-clonic***. Less frequently, *myoclonic, atonic or absence* seizures can occur.

- ***Tonic-clonic seizures*** are the most common type of generalized-onset seizure in dogs (Berendt and Gram, 1999; Licht et al., 2002). The prodromal phase and the aura is not always recognized by the owner. The ictal phase is characterized by generalized contraction of the body muscles resulting in a rigid extension of the limbs and opisthotonos, usually lasting 10 to 60 s (tonic phase). The animal falls on its side and often loses consciousness. Breathing is frequently irregular and the animal might become cyanotic. The tonic phase is followed by the clonic phase, which is characterized by rhythmic muscular contractions resulting in jerking movements of the limbs. These stages are often associated to autonomic signs, such as hypersalivation, urination, defecation and mydriasis (De Risio, 2014a).
- ***Tonic seizures*** are characterized by an increased muscle contraction without clonic motor activity. During this type of seizure the consciousness might impaire and autonomic manifestations might be present (De Risio, 2014a).
- ***Clonic seizures*** consist in a motor activity with no tonic component (Thomas, 2010).
- ***Myoclonic seizures*** are characterized by sudden, brief, involuntary, shock-like contractions that can be generalized (Potschka et al., 2013).
- ***Atonic seizures*** are characterized by a sudden and generalized loss of muscle tone, usually appearing as episodes of collapse (Berendet et al., 2015).
- ***Absence seizures*** are characterized by a transient and brief impairment of consciousness (Poma et al., 2010).

	Early terminology	Terminology currently in use	Suggested veterinary terminology 2015
EPILEPTIC SEIZURES			
An epileptic seizure with clinical signs indicating activity which starts in a localised area in the brain -Will present with focal motor, autonomic or behavioural signs alone or in combination	Petit Mal Aura	Partial/focal seizure - Simple partial/focal seizure (consciousness unimpaired[a]) - Complex partial/focal seizure (consciousness impaired[b])	Focal epileptic seizure[a]
An epileptic seizure with clinical signs indicating activity involving both cerebral hemispheres from the start. -In dogs and cats the seizure presents predominantly as immediate 'convulsions' and loss of consciousness. Salivation, urination and/or defecation often also occur during convulsions. May also (but rare) present as atonic or myoclonic seizures	Grand Mal (always implicating convulsions)	Primary generalized seizure	Generalized epileptic seizure
An epileptic seizure which starts in a localized area in the brain and spreads subsequently to involve both hemispheres. -In dogs and cats the seizure starts with localized motor, autonomic and/or behavioural signs rapidly followed by convulsions. Salivation, urination and/or defecation often also occur during convulsions.	Partial seizure with secondary generalization (secondary generalized seizure)	Focal seizure with secondary generalization	Focal epileptic seizure evolving to become generalized
EPILEPSY			
Epilepsy classified by aetiology	Primary Epilepsy - Epilepsy where no structural cerebral pathology is suspected	Idiopathic Epilepsy - Epilepsy where no structural cerebral pathology is suspected. A genetic component may be involved	Idiopathic Epilepsy 1. Proven genetic background 2. Suspected genetic background 3. Unknown cause and no indication of structural epilepsy
Epilepsy classified by aetiology	Secondary or Acquired epilepsy - Epilepsy caused by identified cerebral pathology	Symptomatic Epilepsy - Epilepsy caused by identified cerebral pathology	Structural epilepsy - Epilepsy caused by identified cerebral pathology
Epilepsy classified by aetiology	Cryptogenic - Meaning hidden	Probably or possibly symptomatic epilepsy - A suspected symptomatic cause, which however remains obscure	Unknown cause

Veterinary terminology and its most common amendaments over time (from Berendet et al., 2015).

1.3.4 PHASES OF GENERALIZED SEIZURES

Generalized seizures are tipically characterized by four phases: prodrome, aura, ictus and post ictal phase. The ***prodrome*** is a period that may occur within hours preceding the ictal phase. During the prodromic phase, the animal displays altered behavior: may hide, appear nervous, or seek out their owners (Lorenz et al., 2011).

The ***aura*** is the initial manifestation of a seizure, lasting usually just a few seconds. Aura is described in people as a subjective sensation, such as dizziness, tingling, and anxiety. In animals aura may be recorded as an increased or decreased attention seeking, stereotypical sensory or motor behaviour (e.g. licking, pacing) or autonomic manifestations (e.g. salivating, vomiting, urinating) (Berendt et al., 2015). The definition of aura in veterinary medicine has generated several controversies. Currently, the aura is considered the focal-onset phase of a secondary generalized seizure.

The ***ictus*** is the seizure itself, reflecting the paroxistic activation of neurons and, according to the semiology, may consist of generalized epileptic seizure, a focal epileptic seizure, or a focal epileptic seizure evolving into a generalized seizure.

The ***postictal phase*** may be absent, short or lasting several hours to days depending on the number and severity of the seizures experienced bu the patient. Typically, the animal is disoriented, may have behavioural abnormalities such as repetitive vocalisation, compulsive locomotion, bumping into obstacles. Furtehrmore, owner descriptions of post ictal phases include ataxia, excessive hunger or thirst, urination, defecation or exhaustion and excessive sleep. Postictal blindness or aggression may also be present (Berendt et al., 2015).

1.4 APPROACH TO EPILEPTIC DOG

The recognition of a seizure in canine and feline patients is sometimes challenging, since in most cases is based upon owners' observation. A detailed and accurate history is essential to identify an epileptic seizure and distinguish disorders mimicking seizure activity (Moore, 2013). Questions to the owners are aimed to understand if the patient has really experienced an epileptic seizure. If the description of the event is vague or difficult to interpret, it's helpful to ask the owner to make a video of the paroxysmal episode (Gandini, 2015a).

The signalment itself may give clues about seizure aetiologies. Generally, puppy and young animals are more likely to develop infective and congenital diseases. Conversely, elder patients suffer more often from neoplastic lesions. Dogs with seizure onset between 6 month and 6 years, especially some canine breeds, commonly suffer from idiopathic epilepsy (De Risio, 2014b).

The collection of information about the signalment might be followed by a detailed medical history. The owner should describe the episode, reporting its duration and frequency.

He should tell if the events occur at a certain time of the day and/or in association with specific situations, such as feeding or exercise, and if there are any interictal abnormalities, including changes in behavior, gait, appetite, weight, or sleep habits.

Furthermore, the physician should focus on previous medical history, including earlier occurrence of head trauma, febrile disorder, vaccination diet, exposure to toxins.

A careful phisical examination should precede the neurological examination. By doing so, illness underlying the cause of seizures or abnormalitis of cardiac or respiratory origin that might mimic a seizure, could be detected (Thomas and Dewey, 2016).

The neurological examination must be complete, including the evaluation of mentation, gait and posture, cranial nerves, postural reactions, spinal reflexes and spinal palpation. The neurological examination is focused to identify clinical signs suggesting a structural disease (Moore, 2013).

According to the signalment, age at seizure onset, presence or absence of other clinical abnormalities (in addition to the seizures), onset, course and distribution of the other neurological abnormalities (if present), the clinician produces a list of differential diagnosis and chooses the most appropriate diagnostic tests (De Risio, 2014b).

A complete blood count, a serum biochemistry analysis should be always performed to rule out metabolic causes and to obtain information about the patient's metabolic status useful for a possible diagnostic anesthesia and for therapy. Magnetic resonance imaging (MRI) is the diagnostic imaging modality of choice for the evaluation of the brain in animals with seizures. MRI is indicated any

time structural epilepsy is suspected and, in case of a normal scan, to support the diagnosis of idiopathic epilepsy (De Risio, 2014b; Gandini, 2015a).

The cerebrospinal fluid (CSF) analysis is recommended when an inflammatory/infectious cause of seizure is highly suspected. Survey radiography of the thorax and ultrasonography of the abdomen should be performed in animals with suspected neoplastic disease. Abdominal ultrasound is also indicated to investigate certain metabolic disorders causing seizures such as insulinoma-associated hypoglycaemia and hepatic encephalopathy due to a portosystemic shunt (De Risio, 2014b).

To properly approach a dog with suspect idiopathic epilepsy, IVETF has recently described a three-tier system, progressively more accurate in establishing the presence of IE (De Risio et al., 2015).

According to the ***tier I confidence level*** the diagnosis of IE occurs in case of a history of two or more unprovoked epileptic seizures happening at least 24 h apart, with an age at epileptic seizure onset of between 6 months and 6 years, an unremarkable interictal physical and neurological examination, and no significant abnormalities on minimumdata base (MDB) blood tests and urinalysis. The MDB blood tests should include: complete blood cell count and serum biochemistry profile (sodium, potassium, chloride, calcium, phosphate, alanine aminotransferase - ALT), alkaline phosphatise (ALP), total bilirubin, urea, creatinine, total protein, albumin, glucose, cholesterol, triglycerides, and fasting bile acids and/or ammonia. Urinalysis should include specific gravity, protein, glucose, pH, and sediment cytology. Additional tests vary according to the differential diagnosis.

The ***tier II confidence level*** includes unremarkable fasting and postprandial bile acids, brain magnetic resonance imaging (MRI), and cerebrospinal fluid (CSF) analysis. If the results of routine CSF analysis are abnormal additional test on CSF for infectious diseases should be performed.

The ***tier III confidence level*** includes, on electroencephalography, the identification of characteristic electroencephalographic abnormalities for seizure disorders.

1.4.1 MAIN CLINICAL FEATURES OF IDIOPATHIC EPILEPSY

Idiopatic epilepsy (IE) is the most common cause of epilepsy in dogs (Thomas and Dewey, 2016,). The prevalence of IE has been extimated varying from 0.5 to 5% in the general canine population (Thomas, 2016; Platt and De Risio, 2014).

In several breeds the prevalence documented is much higher. For example in the Belgian Shepherd Tervueren has been reported to be 9,5%, in the Irish Wolfhounds 18,3%, in the Border Terrier 13,1%, in the Petit Basset Griffon Vendeen 8,9%, in the Labrador Retriever 3,1%, in the Finnish Spits Dog 5,4 %, in the Spinone Italiano 5,3 % (Berendt et al., 2008; Casal et al., 2006; Kloene et al., 2008; Gullov et al., 2011; Berendt et al., 2002; Vitmaa et al., 2013; De Risio et al., 2015a). This higher rate in some breeds compared to the general population, is one of the reasons why a genetic component is highly suspected (Hülsmeyer et al., 2015).

Most dogs with IE suffer their first seizure between 6 months and 6 years of age, although seizures occasionally start before 6 months or as late as 10 years of age (Podell et al., 1995; Heynold et al., 1997; Jaggy and Bernardini, 1998; Berendt and Gram, 1999; Thomas, 2010).

In the past, generalized tonic-clonic seizures were considered the most common type of seizure in dogs with idiopathic epilepsy (Heynold et al., 1997). However, more recent studies have shown that the most common seizure type in dogs with IE is the focal seizure with secondary generalization (Platt and De Risio, 2014). Dogs with IE may present cluster seizures (CS) and/or status epilepticus (SE). According to previous studies, the frequency of idiopathic epileptic dogs suffering CS varies from 41 to 49%, the SE range from 2,5-15% (Monteiro et al., 2012; Packer et al., 2016). Several breeds seem to be predisposed to CS. In Monteiro study, German Shepherd Dogs and Boxers were found more likely to suffer from CS than Labrador Retrievers (Monteiro et al., 2012). On the other hand, a more recent study has confirmed the predisposition of Border Collie to CS, as previously reported, and has identified further breeds, including the Cavalier King Charles Spaniel and the Staffordshire Bull Terriers (Hülsmeyer et al., 2010; Packer et al., 2016).

The diagnosis of IE is made by ruling out other possible causes and is based on the age at epileptic seizure onset, unremarkable inter-ictal physical and neurological examinations, and the exclusion of metabolic, toxic and structural cerebral diseases.

1.4.2 MAIN CLINICAL FEATURES OF STRUCTURAL EPILEPSY

Structural epilepsy is a condiction characterized by repeated seizures due to a known and identifiable structural forebrain disorder such as vascular, inflammatory/infectious, traumatic, anomalous/developmental, neoplastic and degenerative diseases (De Risio, 2014c).

According to previous studies, the prevalence of structural epilepsy in dogs and cats ranges from 25–38% in dogs and 34–87% in cats (Quesnel et al., 1997; Bateman and Parent, 1999; Platt and Haag, 2002; Pákozdy et al., 2008; Schriefl et al., 2008; Zimmermann et al., 2009; Steinmetz et al., 2013; De Risio, 2014c).

Animals with structural epilepsy usually present an abnormal interictal neurological examination. They could show prosencephalic signs, such as disorientation, aggression, compulsive walking, head pressing, and circling.

During a posture examination, clinicians might sometimes observe pleurothotonus (usually ipsilateral to the affected forebrain side).

Tipically, gait evaluation does not identify remarkable abnormalities, but propriocetive deficits could be present. On cranial nerves examination, the only alteration detected might be the menace response and to cotton ball tests (De Risio, 2014c; Gandini; 2015a).

However, focal lesions in "clinically silent" areas of the brain (including olfactory bulb, frontal, and pyriform lobes) can result in seizure activity without any other neurological sign (Foster et al., 1988; Smith et al., 1989, De Risio et al., 2015b). In a study evaluating the risk factors for development of epileptic seizures in dogs with intracranial neoplasia, epileptic seizure was the first sign noted by the owners in 76 % of dogs (Schwartz et al., 2011).

The combination of information concerning signalment, history, disease onset and course help to formulate the differential diagnosis list. On the basis of the differential diagnosis, the physician will choose the most appropriate diagnostic investigations. For those patients in which structural epilepsy is highly suspected an MRI and CSF analysis should be strongly recommended and represent the core of the diagnostic work-up (Moore, 2013)

Treatment of structural epilepsy is aimed to treat the underlying aetiology and control the seizures with antiepileptic medications (De Risio, 2014c; Moore, 2013).

1.4.3 MAIN CLINICAL FEATURES OF REACTIVE SEIZURES

Reactive seizures are the reaction of a normal brain to a systemic metabolic, nutritional or exogenous toxic disorder (Podell et al., 1995). They can result from a variety of metabolic disturbances (e.g., hypoglycaemia, electrolyte disorders, portosystemic shunt resulting in hepaticvencephalopathy) or intoxications (e.g., carbamates, organophosphates, lead poisoning,

ethylene glycol toxicity, metaldehyde, strychnine) (De Risio et al., 2015b).

Clinical presentation in animals affected by metabolic/toxic disorders is variable depending on the underlying aetiology (De Risio, 2014d). Toxic disorders tipically have an acute (< 24 h) onset and neurological signs may be preceded or accompanied by gastrointestinal, cardiovascular or respiratory signs. Metabolic disorders can present with an acute, subacute, or chronic onset and may be progressive or characterized by waxing and waning signs. Systemic clinical abnormalities can often be detected on general physical examination. The neurological examination generally reveals diffuse, bilateral and often symmetrical neurological deficits, however seizures can sometimes be the only neurological abnormality (De Risio et al., 2015b).

In a study investigating metabolic and toxic causes of canine seizures, the most frequent cause of reactive seizures were intoxications (39 %, 37/96 of dogs) and hypoglycaemia (32 %, 31/96 of dogs). Metaldehyde (19%, 7/37) and organophosphate or carbamate poisoning (16%, 6/37) were the most frequent intoxications (Brauer et al., 2011). In this study, 41 % (39/96) of dogs were presented in status epilepticus (Brauer et al., 2011). According to another study, dogs with reactive seizures have a significantly higher risk of developing status epilepticus, particularly as first manifestation of a seizure disorder, than dogs with other seizure aetiologies (Zimmerman et al., 2009).

For patients in whom a reactive cause of seizures is suspected, initial diagnostic workup should include a complete bloodcount, chemistry profile (including glucose and electrolytes), measurement of preprandial and post- prandial bile acids, and urinalysis (Moore, 2013).

Most of these conditions are reversible depending on the underlying disease; therefore permanent antiepileptic drug therapy should only be initiated when the seizures are uncontrolled despite therapy or when an emergency situation such as status epilepticus occurs (Boggs, 1997).

1.5 TREATMENT OF EPILEPSY

In veterinary medicine, the mainstay of epilepsy treatment is the administration of antiepileptic drugs (AEDs). Seizure eradication in dogs is a target unlikely to achieve. More realistically, the main goal is the reduction of seizure frequency, duration and severity with limited and acceptable AED adverse effects to maximize the dog and owner's quality of life (Bhatti et al., 2015).
This goal should be clearly explained to the pet owner to avoid non-commensurate expectations.
A good compliance with the owner is the key to a successfull treatment (Gandini, 2015b).
To date, there is no evidence-based data on when to start antiepileptic treatment in dogs (De Risio, 2014e). The decision is taken on a case-by-case basis, considering information about the general health of the patient, the owner's lifestyle, financial limitations and comfort with the proposed therapeutic regimen (Muñana, 2013). Recently, a consensus statment on seizure management in dogs was produced on the basis of a careful meta-analysis of all the literature concerning seizures and epilepsy including the proceedings of Annual Congresses of the European Society and College of Veterinary Neurology (ESVN / ECVN) and the American College of Veterinary Internal Medicine (ACVIM)[(Podell et al., 2016)].
According to the careful meta analysis of the literature, the "2015 ACVIM Small Animal Consensus Statement on Seizure Management in Dogs" has established guidelines for a predetermined, concise and logical sequential approach to seizure management, reviewing decision-making, treatment strategies, focusing on issues related to chronic AED treatment response and monitoring, and concluding with guidelines to enhance patient response and quality of life (Podell et al., 2016).
The "2015 ACVIM Small Animal Consensus Statement on Seizure Management in Dogs", in agreement with the International Epilepsy Veterinary Task Force statements, has suggested starting the treatment when the animal has:

- Identifiable structural lesion or a prior history of brain disease or injury
- Interictal period of ≤ 6 months (i.e. 2 or more epileptic seizures within a 6 month period)
- Status epilepticus or cluster seizures
- Particularly severe postictal signs (e.g. aggression, blindness) or lasting longer than 24 hours
- An increased epileptic seizure frequency and/or duration and/or deterioration over 3 interictal periods of seizure severity (Bhatti et al., 2015, Podell et al., 2016).

Concerning the choice of the AED, several factors should be taken into account: *AED-specific factors* (e.g. regulatory aspects, safety, tolerability, adverse effects, drug interactions, frequency of administration), *dog-related factors* (e.g. seizure type, frequency and aetiology, underlying pathologies such as kidney/hepatic/gastrointestinal problems) and *owner-related factors* (e.g. lifestyle, financial circumstances) (Bhatti et al., 2015).

Until recently, primary treatment options for dogs and cats with epilepsy were limited to phenobarbital and bromide. In 1857, Sir Charles Locock used bromide to treat "hysterical" epileptic fits in women. Phenobarbital was discovered in 1912 (Podell, 2013). Although these two drugs are still the most widely used in veterinary practice, over the past 20 years in human medicine several new antiepileptic drugs have been developed. Some of them are now used in the treatment of canine and feline epilepsy. According to their appearance, they have been categorized into first, second, third and next generation AEDs (Podell, 2013).

Among the first generation AEDs, the most used in veterinary medicine are: bromide, phenobarbital and benzodiazepines.

The second generation AEDs include Zonisamide, Levetiracetam, Topiramate, Gabapentin and Pregabalin. In 2013, a new AED, the Imepitoin, was introduced in Europe for the treatment of generalized-onset seizures in idiopathic epileptic dog (Tipold et al, 2015).

Old generation		New generation	
First generation (1957–1988)	Second generation (1989–2007)	Third generation (2008–2009)	Next generation (2010–2013)
Bromide	**Felbamate**	**Lacosamide**	**Brivaracetam**
Phenobarbital	**Gabapentin**	**Rufinamide**	**Imepitoin (ELB138)**
Benzodiazepines	**Zonisamide**	Eslicarbazepine acetate	Carisbamate
Primidone	**Levetiracetam**		Flurofelbamate
Phenytoin	**Pregabalin**		Tiagabine
Carbamazepine	**Topiramate**		Losigamone Retigabine
Valproate	Lamotrigine		Remacemide
Ethosuximide	Oxcarbazepine		Seletracetam
	Vigabatrin		

Anti-epileptic drugs categorized by generation of development (form De Risio, 2014e).

The 2015 ACVIM consensus, after the careful evaluation of the literature identified 4 levels of recommendation based on the evidence of efficacy for the first-line treatment of IE. According to the data resulting from the meta-analysis, Phenobarbital and Imepitoin are highly recommended for

the first-line antiepileptic monotherapic treatment. Bromide has moderate scientific evidence and levetiracetam and zonisamide have poor evidence to be recommended as first-line drugs used in monotherapy for the treatment of IE.

At the onset of the treatment is recommended the use of a single AEDs rather than a combination of drugs. The monotherapy improves the compliance and limits the cost, the adverse effects and pharmacokinetic/dynamic interactions. If seizures are not very frequent, the anti-epileptic treatment can be started at the lower dose and increased gradually based on efficacy, tolerability and, for those drugs requiring it, serum concentration monitoring. Conversely, dogs with frequent and severe seizures should be administered a loading dose to achieve quickly the therapeutic level (De Risio, 2014e).

The decision to add a second AED is based-on seizures frequency, severity (duration, cluster activity, postictal effects), and overall quality of life. Strict criteria for decision-making strategy on starting a second AED are lacking in veterinary medicine. Several factors should be considered when deciding on a second AEDs. Selection of an AED with a different mechanism of action, minimizing drug-drug interactions, avoiding additive toxicity, and determination of risk-benefit of polypharmacy versus quality of life are important considerations (Podell et al., 2016). The 2015 ACVIM consesus reported good evidence to recommend phenobarbital, bromide, zonisamide and levetiracetam as add-on AEDs and imepitoine as not reccomanded (Podell et al., 2016).

Table 1. ACVIM panel recommendations of AED use, monitoring, and risk profile.

	Monotherapy recommendation			Risks Types				Add-on AED recommendation	
Drug	Level	Grade	Monitor drug levels	1	2	3	4	Level	Grade
Phenobarbital	I	A	Y	Y	Y	Y	N	IV	B
Bromide	I	B	Y	Y	Y	Y	N	II	B
Primidone	II	D	Y	Y	Y	Y	N	II	D
Imepitoin	I	A	N	Y	N	N	N	III	C
Levetiracetam	IV	C	N	Y	N	N	N	Ib	B
Zonisamide	III	C	Y	Y	Y	N	N	III	B

Level of study design

Level I and Ib Appropriately designed, controlled trials

1 Criteria

a I: Blinded, randomized clinical trials and drug efficacy of ≥50% for at least 6 months

b Ib: Blinded, randomized clinical trials and drug efficacy of ≥50% for less than 6 months

Level II: Case-control or cohort studies

1 Criteria:

a Nonblinded, randomized, or nonblinded and nonrandomized clinical trials with cohort size of 15 or more, drug efficacy of > or equal to 50% for > 12 weeks, or both.

Level III: Case reports or series

1 Based on individual case reports, conference proceedings, and/or other media distribution as a *potentially* effective and/or predictable outcome

2 Criteria:

a Nonblinded and nonrandomized clinical trials with cohort size of less than 15 and/or drug efficacy of ≥50% for >12 weeks

Level IV: Expert opinion only

1 Based on any level of scientific information as an unestablished, ineffective, and/or harmful

2 Criteria: Expert opinion only without documentation of cohort studies

Grade of ACVIM panel recommendation

1 A: High recommendation and likely be effective treatment

2 B: Moderate recommendation and most likely to be effective treatment

3 C: Low recommendation and may not be effective treatment

4 D: Not recommended for treatment and may be ineffective and/or dangerous to the patient

From 2015 ACVIM Consensu statement on seizure Management in dogs (Podell et al., 2016).

1.5.1 PHENOBARBITAL

Phenobarbital (Pb) has the longest history of chronic use of all AEDs in veterinary medicine (Podell et al., 2016). It is generally the first drug of choice in epileptic dogs and cats (De Risio, 2014f).

PB is reported to be effective in reducing or eradicating seizure activity in 60 to 85 per cent of dogs with idiopathic epilepsy, if serum concentration is maintained within the therapeutic range (Farnbach, 1984; Morton and Honhold, 1988; Schwartz-Porsche et al., 1985; Boothe et al., 2012). The superior efficacy as a first-line antiepileptic drug in dogs was demonstrated in a randomized controlled trial comparing to bromide, in which 85% of dogs treated with phenobarbital became seizure-free compared with 52% of dogs treated with potassium bromide.

Pb enhances the postsynaptic inibition increasing responsiveness inhibitory neurotransmitter (GABAA receptor). Specifically, it enhances receptor-mediated chloride currents by prolonging the opening of postsynaptic chloride channels, resulting in increased intracellular chloride concentration and subsequent hyperpolarization of the cell membrane. At higher concentrations, it also causes a presynaptic reduction of calcium dependent action potentials, which might also contribute to its antiepileptic effect (Muñana, 2013).

It is rapidly absorbed after oral administration in dogs and achieves the peak serum concentrations approximately 4− 8h after oral administration in dogs (Ravis et al., 1984).

The elimination half-life in normal dogs has been reported to range from 37− 73hours after multiple oral dosing (Ravis et al., 1984). Plasma protein binding is approximately 45% in dogs (Frey et al., 1979). PB is metabolized primarily by the liver and approximately 25% is excreted unchanged in the urine. PB is a potent inducer of cytochrome P450 enzyme activity in the liver (Hojo et al., 2004) and, for this reason, is contraindicated in dogs with hepatic dysfunction. The induction of cytochrome P450 activity leads to increased clearance resulting in the reduction of PB serum concentrations and possible therapeutic inadequacy. Therefore, monitoring of serum PB concentrations is mandatory for proper dose modulation over time (Bhatti S et al., 2015).

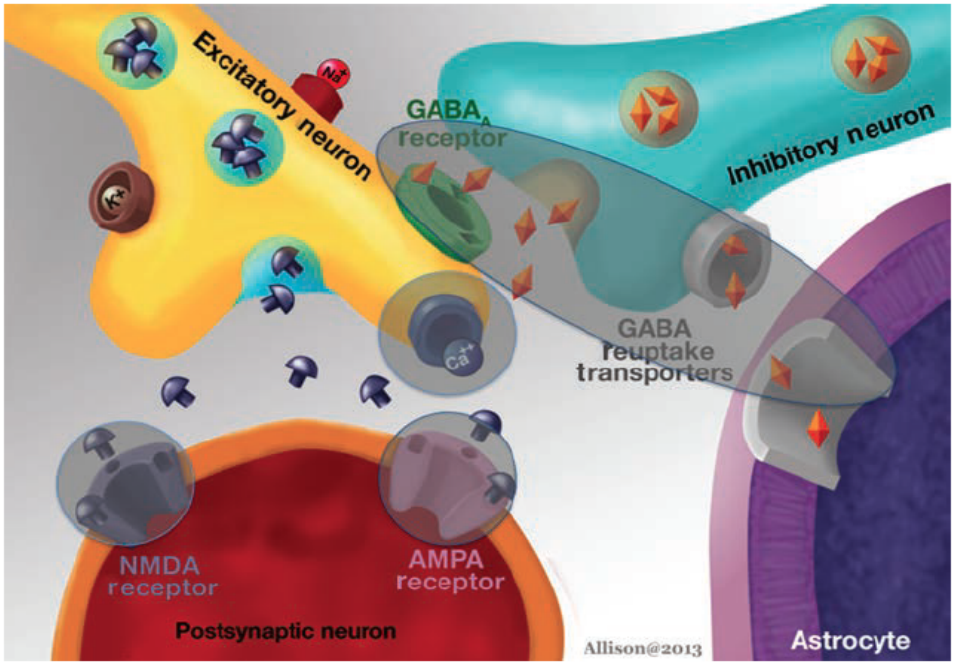
Simultaneous PB administration with other drugs metabolized by cytochrome P450 subfamilies and/or bound to plasma proteins, can alter drugs pharmacokinetics and, as a consequence, may decrease the therapeutic effect of other AEDs (levetiracetam, zonisamide, and benzodiazepines) as well as corticosteroids, cyclosporine, metronidazole, voriconazole, digoxin, digitoxin, phenylbutazone and some anaesthetics (Bhatti S et al., 2015). Conversely, the co-administration of PB with drugs that inhibit the cytochrome P450 such as cimetidine, omeprazole, lansoprazole, chloramphenicol, trimethoprim, fluoroquinolones, tetracyclines, ketoconazole, fluconazole,

itraconazole, fluoxetine, felbamate and topiramate may inhibit PB metabolism increasing the serum concentration (Bhatti S et al., 2015).

The recommended oral starting dose of PB in dogs is 2.5− 3 mg/kg BID. The serum concentration should be measured 14 days after starting therapy once steady state concentrations are achieved ad after the change of the dose.

The therapeutic range of PB in serum is 15 mg/l to 40 mg/l (Bhatti S et al., 2015).

Most of the adverse effects due to PB are dose-dependent, occur at the beginning of the treatment or after an increase in the dose and, generally, disappear or decrease in the subsequent weeks. The most frequent adverse effects are: sedation, ataxia, polyphagia, polydipsia and polyuria (Podell et al., 2016). Less frequently, the administration of Pb may lead to idiosyncratic reaction including hepatotoxicity (Bunch et al., 1982; Dayrell-Hart et al., 1991; Gaskil and Cribb, 2005; Müller et al., 2000), haematologic abnormalities (anaemia, and/or thrombocytopenia, and/or neutropenia) (Jacob et al., 1999; Bersan E., 2012), superficial necrolytic dermatitis (March PA et al., 2004), potential risk for pancreatitis (Gaskill and Cribb, 2000), dyskinesia, anxiety (Kube et al., 2006) and hypoalbuminaemia (Gieger et al., 2000). Usually they resolve after therapy discontinuation (Bhatti S et al., 2016).



Neuronal receptor targets for phenobarbital (from De Risio, 2014f).

1.5.2 IMEPITOIN

Imepitoin is a new AED approved in the Europe in 2013 for the treatment of canine idiopathic epilepsy.

It was initially developed for the treatment of anxiety and epilepsy in man, but, then, it has not been used in humans because in smokers it had a different pharmacokinetic (Rundlfeldt et al., 2015).

Based on promising findings of imepitoin in a preclinical seizure model in dogs, it was decided to develop this drug for the treatment of canine epilepsy (Loscher et al., 2004). Imepitoin acts as a low affinity partial agonist at the benzodiazepine site of the GABAA receptor (Rundlfeldt and Loscher, 2014).

It is administered orally and achives the maximal plasma levels after 2− 3. The elimination half-life is approximately 1.5 to 2 hour. It is metabolized mainly in the liver (Podell et al. 2016). There is no information on pharmacokinetic interactions between imepitoin and other drugs (De Risio, 2014m).

The most common adverse effects reported include: ataxia and polyphagia, followed by sedation, hyperactivity, increased serum creatinine activity, vomiting and diarrhoea, disorientation and polydipsia. Less commonly, polyuria, anxiety, tachypnea, hypersalivation, decrease in sight and motor activity, prolapsed nictitating membrane and increased sensitivity to sound (Charalambous et al., 2016). Recently, has been described a cutaneous adverse reaction associated with chronic administration of imepitoin in a Jack Russel terrier (Royaux et al., 2016).

The IVETF recommend starting the treatment with imepitoin at the dose of 10− 20 mg/kg q 12 hours. If seizure control is not satisfactory after at least 1 week of treatment at this dose, it can be increased up to a maximum of 30 mg/kg q 12 hours. Reference range of plasma or serum imepitoin concentrations is unknown and there are no therapeutic monitoring recommendations for imepitoin (Bhatti et al., 2015).

1.5.3 BROMIDE

Bromide (Br) is a salt used the first time for the treatment of human epilepsy in 1857 (Locock, 1857). It is thought to exert its antiepileptic activity by passing through neuronal chloride ion

channels with subsequent neuronal hyperpolarization, raising the seizure threshold and stabilizing neurons against excitatory input from epileptic foci (Baird-Heinz et al., 2012).

After oral administration, the bioavailability of Br is approximately 46 % **(**Trepanier and Babish, 1995**)**. Due to its long elimination hal-flife the steady state is reached approximately 2.5–3 months after treatment initiation at maintenance dosage (Podell and Fenner, 1993; Trepanier and Babish, 1995; Podell, 1998; Ducoté, 1999; March et al., 2002).

Br is not metabolised in the liver, therefore is a good option in dogs with hepatic dysfunction (Bhatti et al., 2015). It is excreted unchanged by the kidneys, where it is filtered by the glomeruli and then undergoes tubular reabsorption in competition with chloride (Dewey, 2006). An increase intake of chloride leads to increase the renal elimination of Br with subsequently decrease of serum concentration and potentially loss of seizure control (Baird-Heinz et al., 2012).

On serum chemestry the chloride concentrations are often falsely increased because the assays cannot distinguish between chloride and Br ions (Trepanier, 1995)

Common adverse effects of Br in dogs include sedation, ataxia and pelvic limb weakness, polydipsia/polyuria, and polyphagia with weight gain (Baird-Heinz et al., 2012; Dewey, 2006; Podell et al., 2016). These adverse effects seem to be dose-dependent. Commonly, they occur at the biginnig of treatment and partly or completely subside after the achivement of the steady-state concentrations (De Risio, 2104g). Potassium bromide is a mucosal irritant, and it may cause nausea, vomiting and/or diarrhoea. In order to prevent gastrointestinal irritation it is prefered administer Br with food and share the daily dose into two or more doses (Baird-Heinz et al., 2012). Uncommonly adverse effects are personality changes (like aggressive behaviour, irritability, and hyperactivity), persistent cough, increased risk of pancreatitis and megaoesofagus (Bhatti et al., 2015). The recommended starting dose of Br is 15 mg/kg q 12 hours when used as adjunctive therapy, 20 mg/kg q 12 hours when used as a monotherapy (Bhatti et al., 2015; De Risio, 2014g). The reported therapeutic range is 810 mg/l - 2000 mg/l when administered with phenobarbital, 2000mg/l to 3000mg/l when administered alone (Podell et al., 2016). To reach more rapidly the steady state concentration, due for instance to severe seizures, or because phenobarbital must be rapidly discontinued due to life-threatening adverse effects, it is possible to administer a loading dose of Br. The IVETF suggests two protocols: 625 mg/kg given over 48h and divided into eight or more doses or 125 mg/kg/day divided in three to four daily administrations for 5 consecutive days (Bhatti et al., 2015).

1.5.4 BENZODIAZEPINES

Benzodiazepines exert their anticonvulsant effects by enhancing GABA activity in the brain (Dewey, 2006). In veterinary medicine diazepam is the most used drug especially for the treatment of emergency seizures by intravenous and *per rectum* administration (Podell, 2013).
Diazepam is not used as an oral maintenance anticonvulsant in dogs because of its short halflife of elimination (2–4 hours) and the tendency for dogs to develop tolerance to its anticonvulsant effect (Dewey, 2006). On the contrary, it could be used in cats, but unfortunately its oral use has been associated with acute fatal hepatic (Center et al., 1996; Hughes et al., 1996).
In human, midazolam has been shown to be more effective and safer for the control of seizures than comparable doses of diazepam (Koul et al., 1997; Nordt and Clark, 1997). In dogs and cats, its use is poorly documented. It is used especially in the treatment of status epilepticus. Midazolam can be administered by intravenous bolus, continuous intravenous infusion or intra muscular injection at the raccomanded dose 0,07 - 0,2 mg/kg (Platt, 2014b).

1.5.5 ZONISAMIDE

Zonisamide is a sulphonamide-based anticonvulsant approved for use in humans. It acts with multiple mechanisms of action, including blockage of calcium channels, enhancement of GABA release, inhibition of glutamate release, and inhibition of voltage-gated sodium channels (Bhatti et al., 2015). In dogs, zonisamide is well absorbed after oral administration. It is metabolized predominanltly by hepatic enzime. The elimination half-life is approximately 15hours in dogs (Podell et al., 2016). In people, it has been shown that the elimination half-life of zonisamide is dramatically shorter in patients already receiving drugs that stimulate hepatic enzymes in comparison with patients who are not receiving such drugs. A similar phenomenon seems to occur in dogs (Dewey, 2006).
The recommended starting dose of zonisamide in dogs is 3− 7 mg/kg q 12 hours and 7− 10 mg/kg q 12 hours in dogs in wich inducer hepatic enzymes, such as phenobarbital, are coadminister. In human the terapeutic target range is 10 - 40 μg/ml, it can be used as a guidance regarding effective

concentrations that can be targeted in dogs. Serum concentrations of zonisamide should be measured 1 week after treatment initiation or after every dosage adjustment (De Risio, 2014h). The efficacy on the use of zonisamide in dogs is limited to three studies. The first one valued the efficacy of oral zonisamide as a monotherapy (Chung et al., 2012), while the other two studies evaluated the efficacy as an add-on treatment in dogs with refractory idiopathic epilepsy (Dewey et al., 2004; von Klopmann et al., 2007).

The most common adverse effects reported in dogs include: sedation, vomiting, ataxia, and loss of appetite (Chung et al., 2012; Dewey et al., 2004; von Klopmann et al., 2007). Other adverse effects reported are hepatotoxicity (it has been described in 2 dogs receiving zonisamide monotherapy), renal tubular acidosis (in a dog receiving zonisamide monotherapy), and erythema multiforme (Schwartz et al., 2011; Miller et al., 2011; Cook et al., 2011; Ackermann et al., 2016).

Zonisamide should be used with caution in dogs with renal or hepatic impairment (Bhatti et al., 2015).

1.5.6 LEVETIRACETAM

Levetiracetam was approved for use in 1999 for the treatment of partial-onset seizures in humans (Muñana, 2013). It acts modulating the release of neurotransmitters by binding to the presynaptic vescicle protein (SVA2) (Volk, 2008). It is rapidly absorbed after oral administration and is primarily excreted in the urine (De Risio, 2014i). Levetiracetam has a minimal hepatic metabolism, so it is recommended in animals with hepatic dysfunction. The oral maintenance dose of levetiracetam in dogs is 20 mg/kg q 8-6 hours (Bhatti et al., 2015). In human, the concomitant administration of AEDs inducing cytochrome P450 metabolism (as phenobarbital), may increase the clearance resulting in a lower plasma concentrations (Contini et al., 2004). This effect has also been demonstrated in healthy dogs. In this situation, it is advisable to increase the oral dose of levetiracetam (Moore, 2011).

In veterinary medicine, the serum concentrations of levetiracetam are not routinely measured. The reference range has not been established. As a guidance regarding effective concentrations, the human target range (12− 46 μg/l) can be used (Bhatti et al., 2016).

Levetiracetam is well tolerated and generally safe. Except for mild sedation, ataxia, decreased

appetite and vomiting, adverse effects are very rarely described in dogs (Muñana et al., 2012; Volk et al., 2008).

1.5.7 GABAPENTIN AND PREGABALIN

Gabapentin has been approved for people in Europe since 1993 for adjunctive treatment of focal seizures with or without secondary generalisation and for the treatment of post-herpetic neuralgia (Bhatti et al., 2015).

The precise mechanism of action is unclear, is believed that it acts binding to a specific modulatory protein of voltage-gated calcium channels, resulting in presynaptic inhibition of calcium influx, subsequent inhibition of excitatory neurotransmitter release and attenuation of postsynaptic excitability (De Risio, 2014l). Gabapentin is well absorbed after oral administration, it achives the maximum blood concentration within 2 hours. In dogs, gabapentin is excreted by the kidneys after a partial hepatic metabolism. The elimination half-life is 3-4hours (Muñana, 2013). In litterature there are only two studies that have evaluated the efficacy of oral gabapentin as an adjunct to other AEDs (Govendir et al., 2005; Platt et al., 2006) but none of these have demonstrated an increased likelihood of a successfully response with gabapentin (Charalambous et al., 2016).

The recommended oral dosage of gabapentin in dogs is 10 to 20 mg/kg q 8 hours, although dose reduction may be necessary in patients with reduced renal function. Sedation and ataxia were the most common side effects reported in dogs (Bhatti et al., 2015).

Pregabalin is a GABA analogue that is structurally similar to gabapentin. It was approved in 2004 for the treatment of adults with peripheral neuropathic pain and as adjunctive treatment for adults with focal seizures with or without secondary generalization (Bhatti et al., 2015). In veterinary medicine there is only a study evaluating the efficacy of oral administration of pregabalin as an adjunct to phenobarbital and bromide in dogs. The oral dose in dogs is 3− 4 mg/kgq 12-8 hours. The most common adverse effects reported included sedation, ataxia and weakness (Dewey et al., 2009).

1.5.8 TOPIRAMATE

Topiramate is an AED used in adult and paediatric human patients for the treatment of focal (partial) and generalized seizures (Platt, 2014c). It acts enhancing GABA-ergic activity and inhibiting voltage sensitive sodium and calcium channels, kainate-evoked currents and carbonic anhydrase isoenzymes (Vuu et al., 2016).

In human, topiramate is well absorbed. It is not enterly metabolized, the 70-80 % of an administered dose is eliminated unchanged in the urine (Lyseng-Williamson and Yang, 2007). Also in dogs, topiramate is not enterly metabolized and is primarily eliminated unchanged in the urine. However, a significant biliary excretion is present following topiramate administration (Caldwell et al., 2005).

It has an elimination half-life of 2-4h. The dose of topiramate should be reduced by 50% in patients with renal impairment; instead dosage reductions are not necessary in patients with hepatic impairment. The drug has a relatively low potential for clinically relevant interactions with other medications (Platt, 2014c).

To date, there are no studies about the use of topiramte in monotherapy in dog. Its efficacy has been evaluated as an adjunct to phenobarbital, bromide, and levetiracetam in 10 dogs (Kiviranta et al., 2013). In this study the proposed dose it was recommended to start at a low dosage first (2.0 mg/kg q 12 hours) and increase to a higher dose (5-10 mg/kg q 8-12 hours), in order to minimize the adverse effects. The most common were: sedation, ataxia and weight loss (Kiviranta et al., 2013).

1.5.9 THERAPEUTIC MONITORING OF AEDs

The therapeutic drug monitoring is the measurement, for clinical use, of AED concentrations in body fluids, usually serum (Johannessen and Johannessen Landmark, 2008). The drug monitoring is a mandatory necessity for some first generation AEDs, such as phenobarbital and bromide, to assess:

- the effective drug concentrations after initiation of successful treatment
- if treatment failure is caused by poor compliance or an insufficient or changed drug concentration

- the most proper AED and dosage.
- to prevent toxic effects
- to aid with individualization of treatment (Podell et al. 2016).

It should be measured:

- ✓ After initiating treatment, once steady-state concentrations are achieved
- ✓ After each dosage adjustment, once steady-state concentrations are achieved
- ✓ When seizured are not adequately controlled
- ✓ When there is concern about possible drug-related toxicity
- ✓ At 6- to 12-month intervals to screen for any changes in drug disposition over time (Muñana, 2013).

The therapeutic ranges of drug monitoring represent only an approximation, since they are based-on retrospective data from a small number of patients. Although most responders attain levels within the expected range, some do well below the lower limit whereas others obtain benefit at levels above the upper limit without toxicity (Thomas, 2010).

Therapeutic monitoring of new generation AEDs is not routinely performed and it may be of limited value because there is no established correlation between serum AED concentration and therapeutic efficacy or toxicity (De Risio, 2014e).

1.5.10 DISCONTINUATION OF AEDs

In most cases, the treatment for canine idiopathic epilepsy involves a long-term or lifelong AED administration (Geselle et al., 2015). There are different reasons to discontinue treatment, including the remission of seizures and life-threatening adverse effects. In case of remission of seizures, the decision to gradually taper the dose of an AED should be taken carefully, after a seizure free period of at least 1− 2 years (Bhatti et al., 2015).

In people with prolonged seizure remission (generally 2 or more years), the decision to discontinue AED treatment is done on an individual basis, considering relative risks and benefits (Bhatti et al., 2015). In humans, the chance to remain seizure-free after AED discontinuation is higher in patiens without structural brain lesion, with a short duration of epilepsy, with few seizures before pharmacological control, and with AED monotherapy (O'Dell and Shinnar, 2001; Shih and Ochoa, 2009). In human medicine, a seizure relapse rate ranging from 12% to 67% has been reported after

AED withdrawal (Shih and Ochoa, 2009).

In dogs, there is still little information on risk factors associated with seizure relapse after treatment discontinuation. In a recent paper (Geselle et al., 2015), the consequences of AED withdrawal were studied in dogs with epilepsy. In 11 cases out of 138, the therapy had been stopped after a seizure free period for a median time of 1 year. After therapy discontinuation the majority of these dogs suffered again from seizure (63.6%),

In order to prevent withdrawal seizures or status epilepticus, especially when on PB treatment it is advised to decrease the dose with 20% or less on a monthly basis. In case of life-threatening adverse effects, instant cessation of AED administration under 24h observation is necessary. In these cases, loading with an alternative AED should be initiated promptly in order to achieve target serum concentrations (Bhatti et al., 2015).

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Chapter II

REFRACTORY IDIOPATHIC EPILEPSY

2.1 DEFINITION OF REFRACTORY EPILEPSY

The mainstay in the treatment of canine epilepsy is the administration of AEDs (Bhatti et al., 2015). Nevertheless, according to the data reported on literature, only 15-24% of dogs achieve complete remission (considered as spontaneous or drug-induced seizures freedom) and approximately 75–85% of dogs continue to have seizures during their life (Berendt et al., 2002; Arrol et al., 2012).
Out of this latter population, 20-30% does not attain a satisfactory seizure control neither with two AEDs at adequate dosage (Trepanier et al., 1998; Schwartz-Porsche et al., 1985; Podell and Fenner, 1993). This condition, known as "refractory epilepsy", has been reported with similar incidence in human medicine, (Kwan and Brodie, 2000; Picot et al., 2008). RE represents one of the most important challenges for the clinicians in both humans and dogs. In these species, RE has been associated with highest mortality rates, impaired quality of life and behaviour changes (Kwan and Brodie, 2002; Chang et al., 2006; Berendt et al., 2007; Shihab et al., 2011; Wessmann et al., 2014). In human medicine, several studies have demonstrated the existence of a bidirectional link between the mechanisms underlying the seizure and neurobehavioral comorbidities such as depression, anxiety, and psychosis. These neurobiological disorders enhancing the extent of brain dysfunction increase the probability of developing pharmacorestisant epilepsy (Hitiris et al., 2007; Shihab et al., 2011).
For several years, epilepsy was considered not responsive to the medical management if seizures were not reduced by ≥50% after the treatment with one or two AED(s) used at adequate dose and serum concentration (Regesta and Tanganelli, 1999). A reduction in seizure frequency ≥50% has been used in several clinical studies to establish the therapeutic success (Packer et al., 2014; 2015; Muñana et al., 2012; Dewey et al., 2009; Volk et al., 2008; von Klopmann et al., 2007). However, this threshold has the limitation of considering successfully treated animals experiencing a too high seizure frequency for an acceptable quality of life (Wessmann et al., 2012; Chang et al., 2006). Recently, the IVETF has proposed to apply the definition suggested in 2010 by ILAE, which defined "drug-resistant epilepsy as a failure to achieve seizure freedom despite adequate trials of two (or more) well-tolerated, correctly chosen and used AED regimens" (Kwan et al., 2010). The adaptability of this definition in veterinary medicine is questionable. In the management of canine and feline epilepsy, seizure freedom is the primary treatment goal. However, currently seizure freedom is achieved only in 15-24% of dogs with IE.

For this reason, the IVETF has proposed to distinguish a primary treatment goal, identified with a

"seizure freedom" state and a secondary treatment goal, considered as ***partial therapeutic success***. The "seizure freedom" state was defined as the achievement of a seizure free interval 3 times longest the pre-treatment interictal interval and lasting at least 3 months (Potschka et al., 2015). The additional category of ***partial therapeutic success*** was defined as a relevant reduction in seizure frequency, seizure severity, and the prevention of seizure clusters or *status epilepticus* (Figure 1). In conclusion, the IVETF proposes to apply a modified ILAE Task Force definition for veterinary patients. Thereby, in the IVETF proposal drug-resistant epilepsy should be diagnosed each time seizure freedom is not achieved with two therapeutic trials, indicating if there was evidence for a partial therapeutic success (Potschka et al., 2015). Noteworthy, this new definition lacks an objective threshold and, to date, most papers rely on the former definition of lack of achievemant of a decrease in seizure frequency ≥ 50% despite a treatment with a combination of two or more AEDs at adequate dose.

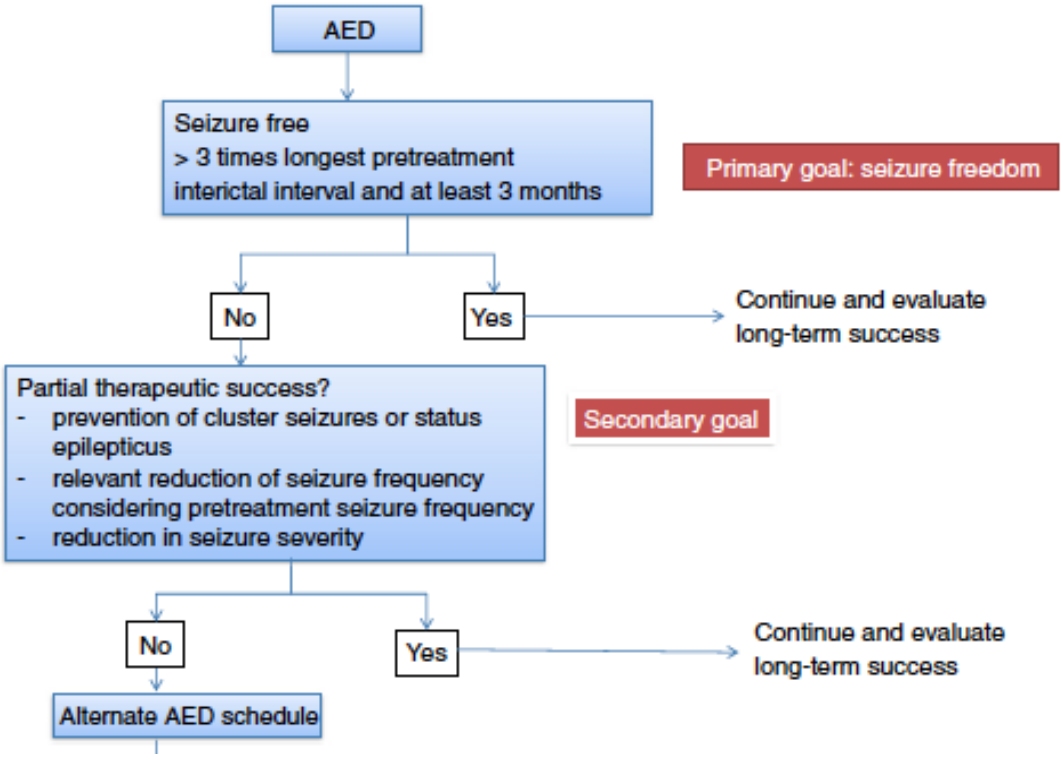

Fig.1 From Potschka et al., 2015

2.1.1 GENETIC FACTORS

RE seems to be a multifactorial disorder involving genetic and acquired factors. The reports of high percentages of IE, particularly severe clinical course and poor response to anti epileptic therapy in specific breeds, such as Border Collies (Hülsmeyer et al., 2010; Arrol et al., 2012; Packer et al., 2014), Australian Shepherd (Weissl et al., 2012) and Italian Spinone (De Risio et al., 2015) raise the possibility of genetic risk factors being responsible for lack of drug responsiveness.

To date, the identification of a causative gene mutation for epilepsy was demonstrated only in the Lagotto Romagnolo affected by a Benign Familial Juvenile Epilepsy (Seppala et al., 2011). The discovery of this mutation and its role is particularly important because it is the first time that a specific genetic mutation was demonstrated to be responsible for a certain type of canine epilepsy (Platt and De Risio, 2014). In this specific case, the mutation is directly linked to remission. The importance of this discovery relies in supporting the hypothesis that similar genetic mechanisms may underlie refractoriness to treatment, especially when faced in specific breeds.

In recent years, specific attention was focused on another gene, the ***ABCB1*** gene encoding the ***P-glycoprotein*** (Kwan and Brodie, 2005). P-glycoprotein is an ATP-dependent transmembrane protein expressed physiologically on the luminal side of the endothelial cells of the Blood Brain Barrier (BBB) that has a protective physiologic function excreting potentially toxic xenobiotics, including AEDs (Basic et al., 2008; Potschka et al., 2002; Mealey, 2004). In Collies and other related breed, the ABCB1 gene may be mutant showing a 4 base pair deletion in exon 4 (c.296_299del) (Mealey et al., 2001).

This mutation, which has a prevalence of >50% in Collies, results in the production of a non-functional P-glycoprotein. The mutated P-glycoprotein does not prevent the normal efflux of substrates from the brain back into the capillary lumen, resulting in central nervous system accumulation of substrate. Therefore, dogs with this mutation have an increased sensitivity to drugs such as ivermectin causing CNS-intoxication (Schinkel et al., 1994, 1996). This defect could also lead to improved seizure control in affected dogs because certain AEDs might penetrate the BBB easier (Muñana et al., 2012). To date, in veterinary medicine only three studies have evaluated the role of ABCB genotype and the response to AEDs.

A study involving 29 Collies with idiopathic epilepsy identified the ABCB1 mutation (c.296_299del) in homozygosis state in 48% of dogs. The dogs with the mutation in homozygosis state had a significantly better seizure outcome than dogs presenting the wild type and the mutation in heterozygosis state (Muñana et al., 2012). Contrarily to this result, in a second study including 50

idiopathic epileptic Australian Sherperd, the mutation in homozygous state, was found only in 2% of cases and an association between seizure control and ABCB1 genotype was not established (Weissl et al., 2012). The third study about the ABCB1 genotype, showed a very low incidence (0,04%) of the mutation (c.296_299del) in the population studied (25 idiopathic epileptic Border Collies). This letter study identified a new mutation in a noncoding promoter region of the gene that was significantly associated with resistance to phenobarbital therapy. It has been hypothesized that an overexpression of these efflux transporters, due to an ABCB1 mutation, may inhibit AED penetration in epileptic foci, resulting in a reduced efficacy of antiepileptic treatment (Alves et al., 2011).

Five potential genes (*KCNQ3*, *SCN2A, GABRA2, EPOX HYD*, and *ABCB4*) associated with response to phenobarbital were identified in a study investigating the polymorphisms in 30 genes involved in drug metabolism, drug targeting, and drug transport. Unfortunately, after correction for multiple comparisons, they did not reach statistical significance (Kennerly et al., 2009). Three of these genes encode ion channels that are targets for AEDs ('*KCNQ3* – voltage gated potassium ion channel important for post excitatory membrane re- polarization, *SCN2A* – sodium ion channel, *GABRA2* – GABA receptor'*)*(Armijo et al., 2005; Volk, 2014). The other two reported genes are involved in phenobarbital metabolism (*EPOX HYD*) or transportation (*ABCB4*) (Kennerly et al., 2009; Volk, 2014). In humans, there is evidence that genetic factors play a major role in epilepsy and, in the most recent update of the nomenclature, the term "idiopathic epilepsy" has been changed with "genetic epilepsy when the gene responsible for the disease has been discovered (Berg et al., 2010).

In Veterinary medicine, while there is clear evidence by pedigree analysis that epilepsy has an hereditary transmission in some breeds, evidence of a specific involvement of certain genes in the pathogenesis of epilepsy (besides the Lagotto Romagnolo) is still lacking (Hülsmeyer et al, 2015). Further studies are necessary to support the hypothesis of a genetic involvement responsible for certain clinical characterisctics, such as drug responsiveness or refractoriness.

2.1.2 CLINICAL FACTORS

In literature, several factors related to the clinical presentation of the disease have been reported to

be involved in the likelihood of successful treatment with AEDs.

In human medicine, it was commonly thought that children treated with AEDs immediately after the first seizure may have an increase likelihood of achieving remission. However, epidemiological studies in developing countries where AEDs are not readily available revealed that remission rates were similar (Placencia et al., 1993). This seems to imply that AEDs are effective in reducing the seizures frequency but have no influence on epileptogenesis (Placencia and others 1993). In veterinary medicine, a study about idiopathic epileptic Labrador Retriever showed that dogs that became seizure-free had a significantly longer interval between first seizure and therapy than those that still had seizures, implying that remission was not influenced by timing of AED medication (Heynold and others 1997). This result was supported by a recent study evaluating clinical risk factors associated with AEDs responsiveness in canine epilepsy, showing no effect of the onset of therapy in achieving a state of full or partial success (Packer et al., 2014). Onset of treatment is likely influenced by disease severity; dogs with a more severe disease course are treated earlier (Packer et al., 2014; Arrol et al., 2012).

In human medicine, a large number of seizures before treatment was a poor prognostic indicator. The patients experiencing a greater number of seizures prior to initiation of treatment were more likely to have refractory epilepsy (Sillanpää, 1993; Kwan 2000; Collaborative Group for the Study of Epilepsy, 1992). In line with these results, in an experimental study on rats, seizure frequency in non-responders to phenobarbital was significantly higher than seizure frequency in responders, demonstrating that high seizure frequency predicts pharmacoresistance in this model (Löscher and Brandt, 2010). The association between a high number of pre-treatment seizures and subsequent refractoriness is likely due to the experimental phenomenon of kindling. Kindling is a phenomenon for which the same repetitive electric stimulation initially at sub-convulsive threshold subsequently becomes sufficient to induce seizures (Raynold, 1993). Also in dogs, some studies associated the large number of seizures before treatment with poor seizure control (Heynolds et al., 1997; Hülsmeyer et al., 2010) Instead, in Packer study (2014) number of seizures before treatment was been found significantly different between dogs with good or poor treatment outcomes. The same study showed that seizure density, considered as the temporal pattern of seizure activity, is a risk factor for the development of AED refractoriness more influential than seizure frequency (Packer et al., 2014)

Early age at onset of seizures acitvity was found to significantly influence the seizure control. Dogs experiencing their first seizure at an older age were more likely to achieve a seizure reduction (Packer, 2014). This was demonstrated also in Border Collies, where the mean age at the onset was significantly higher in dogs with remission compared to those with active epilepsy (Hülsmayer et

al., 2010). Similar findings where shown in a study on Labrador retrievers, where dogs classed as having excellent or good results had a significantly higher age at onset than uncontrolled dogs (Heynolds et al., 1997). In contrast, in a study of canine juvenile epilepsy, the age at onset had no influence on survival outcome (Arrol et al., 2012). In children, the onset of epilepsy before the age of 12 months was considered a poor prognostic factor (Mohanraj and Brodie, 2013).

Ass previously introduced, several studies in canine, rodent and human species show evidence that cluster seizures (seizure density), represent the more influential risk factor on the likelihood of pharmacoresistance in epilepsy rather than seizure frequency or the total number of seizures prior to treatment (Packer et al., 2014; Haut et L., 2005; Löscher and Brandt, 2010).

Seizure type has no significant correlation with refractoriness (Arrol et al, 2012; Volk, 2014). A recent study evaluating the risk factors of drug response in idiopathic epileptic dog, the most common seizure type in dogs not achieving remission was complex-focal seizures (Packer et al., 2014). Similar finidngs are present in human literature, supporting the hypothesis that focal seizures are more challenging to treat (Regesta and Tanganelli, 1999).

Males were found to be less likely to achieve remission than female dogs (Packer et al., 2014).

Another powerful prognostic factor in human medicine is the response to the first antiepileptic drug. Patients with an inadequate response to initial treatment with antiepileptic drugs are likely to suffer from refractory epilepsy (Kwan and Brodie, 2000). A study evaluating the response to successive AEDs in a canine idiopathic epileptic population showed that 123 out 196 (62,8%) were phenobarbital non responders, 59 out 80 dogs receiving phenobarbital and bromide (73,8%) were non responsive and 20 dogs out 32 (37,5%) receiving a third AED had a poor control despite three AEDs (Packer et al., 2015).

2.1.3 PSEUDORESISTANCE

Pseudoresistance is defined as the lack of response due to an inadequate dosing or treatment regime (Potschka et al., 2015). To avoid this condition is pivotal a good compliance with the owner. In case of, doubt regarding the proper administration of phenobarbital or bromide, the clinician should perform the plasma concentration analysis (Potschka et al., 2015). Other reasons of peseudoresistence may depend on pharmacokinetic and dynamic properties of AEDs. For example,

the concurrent administration of phenobarbital alone or together with bromide accelerates the clearance of levetiracetam in epileptic dogs (Muñana et al., 2014). A similar interaction with phenobarbital was shown for zonisamide (Chung et al., 2012; Orito et al., 2008; Potschka et al., 2015). Lastly, as reason of poor seizure control, should be considered the possibility of an identifiable underlying disease process or a misdiagnosis (Volk, 2014). Several conditions may mimic seizures and epilepsy: these include cardiac-associated syncopes, transient vestibular disorders, movement disorders and episodic pain. Sometimes AED treatment can aggravate such mimics (Penning et al., 2009; Volk, 2014).

2.2 MECHANISMS OF REFRACTORINESS

Two major theories have been proposed to explain the pathophysiological mechanisms underlying AED resistance:

1. The drug-target hypothesis:
2. The multidrug transporter hypothesis.

2.2.1 DRUG-TARGET HYPOTHESIS

According to the drug-target hypothesis, drug resistance occurs due to changes in drugs target (I.e. receptors or ion channels) making them less sensitive to AEDs. This hypothesis is based on several studies on voltage-gated sodium channels in hippocampal neurons using carbamazepine (Schdmit and Löscher, 2005). In human patients suffering from temporal lobe epilepsy (TLE) and mesial temporal lobe sclerosis was reported that, in hippocampal neurons, the inactivation on sodium current by carbamazepine is lost (Wreugdenhil et al., 1998). One of the mechanisms to explain the altered sensitivity of sodium channels to carbamazepine is the modification of the subunits composition. Numerous changes in sodium channels expression have been observed both in human

and experimental epilepsy (Bartolomei et al., 1997; Aronica et al., 2001; Whitaker et al., 2001; Ellerkmann et al., 2003). A down regulation of β_1 and β_2 subunit of sodium channel is the likely mechanism explaining the drug resistance (Remy and Beck, 2006).

Apart from modifications in ion channels, other drug target may be altered as GABA receptors.

In a study on a rat model of TLE, alterations of GABA receptors subunit have been associated with resistance to phenobarbital (Bethmann et al., 2008; Volk et al., 2006) and the resistance of status epilepticus to benzodiazepines have been reported to be consequence of GABAA alteration (Macdonald and Kapur, 1999; Chen 2006; Schidmit and Löscher, 2009). To date, no similar studies were performed on canine models.

2.2.2 MULTIDRUG TRANSPORTER- HYPOTHESIS

Drugs enter in the brain passing through the blood-brain barrier (BBB) or traversing the barrier between blood and the cerebrospinal fluid. Because of these anatomical barriers, the entry of drugs into the brain is restricted. The endothelial cells of BBB vessels are connected by tight junctions and surrounded by basement membrane, which is covered with foot processes from astrocytes. Conversely, in choroid plexus the endothelial cells of capillaries are fenestrated and lack tight junctions. However, the epithelial cells lining the surface of choroid plexus are joined by tight junctions, so the permeation barrier at this level is still present (Begley, 2004). The functional consequence is that brain capillaries act in a passive manner restricting the penetration of hydrophilic compounds, and permitting easily the passage of lipid-soluble drugs (Löscher and Potschka, 2002).

However, apart from passive diffusion, drugs may also enter and leave the brain by carrier-mediated transport processes (Löscher and Potschka, 2002). In the last decade, multiple multidrug transporters of the ATP-binding cassette superfamily, especially P-gp and multidrug resistance-associated protein (MRP), have been shown to be expressed physiologically on the luminal side of the endothelial cells of the BBB (Volk, 2014) and in choroid plexus epithelial cells as well (Rao et al., 1999). These transporters appear to act as an active defence mechanism against the penetration of potential CNS toxic lipophilic compounds, therefore limiting the penetration of drugs, such as AEDs (Begley, 2004). The role of multidrug transporters such as PGP or MRPs in

pharmacoresistance has been extensively studied in tumour cells that possess intrinsic or acquired cross-resistance to several chemotherapeutic agents (Tan et al., 2000; Litman et al., 2001). An overexpression of these efflux transporters, inhibiting AED penetration in epileptic foci, may result in a reduced efficacy of antiepileptic treatment (West and Mealey, 2007). This hypothesis is supported by several studies demonstrating a significant higher expression of P-glycoprotein both in epileptogenic brain tissue of human pharmacoresistant patients and in animal models of pharmacoresistant epilepsy (Tishler et al., 1995; Sisodiya et al., 2002; Aronica et al., 2003; Potschka et al., 2004; Volk et al., 2004a, b; Volk and Löscher, 2005; Hoffmann et al., 2006).

Furthermore, it was demonstrated that seizure activity in dogs increases the expression of the multidrug transporter P-glycoprotein in brain capillary endothelial cells (Pekcec et al., 2009).

As previously reported, in veterinary medicine, a polymorphism in the promoter region of ABCB1 gene has been associated with phenobarbital non responsiveness in idiopathic epileptic Border collies (Alves et al., 2011).

Considering this hypothesis, some studies tested the co-administration of an inhibitor of multidrug transporters to current treatment with AEDs. The only P glycoprotein inhibitors that have been clinically evaluated in combination with AEDs in patients with epilepsy have been calcium channel blockers such as verapamil, nifedipine, or diltiazem (Löscher and Schmidt, 1994). Some studies have shown that verapamil can be beneficial in improving seizure control in certain groups of patients with drug-resistant epilepsy and there are reports of efficacy of verapamil in isolated cases of refractory status epilepticus as well (Asadi-Pooyaet al., 2013; Borlot et al., 2014; Nicita et al., 2014; Nicita et al., 2016). However, studies in idiopathic drug-resistant epileptic dogs failed to demonstrate the efficacy of verapamil as add-on treatment in improving seizure control. In this study, verapamil treatment was discontinued in all the dogs due to side effects (e.g., bradycardia and arterial hypotension) or an increase in seizure frequency (Jambroszyk et al., 2011).

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Chapter III

SPERIMENTAL PART

3.1 INTRODUCTION AND AIMS

Idiopathic Epilepsy (IE) is considered the most common chronic neurological disease in dogs (De Risio et al., 2015). IE has an estimated prevalence varying from 0,5 to 5% in the general canine population (Podell et al., 1995; Ekenstedt et al., 2013; De Risio et al., 2015;). In genetically predisposed breeds, the prevalence is much higher (Hülsmeyer et al., 2015). The treatment of canine IE is symptomatic and consists in the administration of anti-epileptic drugs (AEDs) aimed to decrease the seizures frequency and severity (Bhatti et al., 2015). According to the literature, approximately 75–85% IE dogs continue to have seizures (Berendt et al., 2002; Arrol et al., 2012) and, out of this population, 20-30% do not achieve a satisfactory seizure control even when put on treatment with two or more AEDs at appropriate dosages (Trepanier et al., 1998; Schwartz-Porsche et al., 1985; Podell and Fenner, 1993). This condition has been defined Refractory Epilepsy (RE) (Muñana, 2013). Refractory Idiopathic Epilepsy (RIE) represents one of the most important challenges for the human and veterinary neurologists. In human medicine, RIE is considered the cause of highest mortality rates, impaired quality of life and psychosocial disabilities (Kwan and Brodie, 2002). Similarly, dogs with poorly controlled epilepsy have an increased risk of premature death, behaviour changes and a reduced quality of life (Chang et al., 2006; Berendt et al., 2007; Shihab et al., 2011). RIE seems to be a multifactorial condition involving acquired and genetic factors (Volk, 2014; Kwan and Brodie, 2002). In humans, the high seizure frequency before treatment and the inadequate response to initial AEDs treatment were identified as clinical risk factors of refractoriness (Kwan and Brodie, 2000; Mohanraj and Brodie, 2013). In the dog, in a recent study the presence of cluster seizure was considered the main risk factor associated with failure of AEDs responsiveness (Packer et al., 2014). The reports of high percentages of particularly severe clinical course and refractoriness in specific breeds, such as Border Collies (Hülsmeyer et al., 2010; Arrol et al., 2012; Packeret al., 2014), Australian Shepherd (Weissl et al., 2012) and Spinone Italiano (De Risio et al., 2015), support the hypothesis of a genetic influence. The exact pathophysiological mechanisms of RIE are still poorly understood (Stepien et al., 2012). In recent years, specific attention was focused on the role of ATP binding cassette subfamily B member 1 protein (formerly known as multidrug resistance protein 1 or P-glycoprotein) encoded by the *ABCB1* gene (Kwan and Brodie, 2005). P-glycoprotein is an ATP-dependent transmembrane protein expressed physiologically on the luminal side of the endothelial cells of the Blood Brain Barrier (BBB) that has a protective physiologic function excreting potentially toxic xenobiotics, including AEDs. An overexpression of these efflux transporters, due to an ABCB1 mutation, may

inhibit AED penetration in epileptic foci, resulting in a reduced efficacy of antiepileptic treatment (West and Mealey, 2007). In 2011 Alves et al., found that the c.-6-180T>G single nucleotide variation (SNV) of the ABCB1 gene was associated with phenobarbital-resistance in a population of Border Collies (BC) affected by IE (Alves et al., 2011). To date, the presence and relevance of this SNV was not ascertained in other breeds. The early identification of refractoriness and the recognition of the genetic variations underlying drugs unresponsiveness may have relevant clinical and therapeutic implication to improve the outcome and the quality of life of dogs affected by RIE.

For this reason, the aims of the present study consisted in: 1) providing a detailed description of the clinical presentation aimed to identify possible clinical risk factors and 2) assessing the frequency of the *ABCB1* c.-6-180T>G SNV in a multi-breed population of dogs affected by RIE.

3.2 MATERIALS AND METHODS

The present multicentric cross-sectional study investigated the medical records and the blood samples of dogs presented within the period January 2010-December 2014 to six neurological referral centers with a diagnosis of IE.

The diagnosis of IE was based upon the following criteria: onset of seizures between 6 months-6 years of age, normal interictal neurological examination, unremarkable blood results (including complete blood cell count and general biochemical profile) and normal Magnetic Resonance Imaging (MRI) or Computed tomography (CT) findings of the brain. For the purpose of the study, diagnosis of IE in absence of advanced diagnostic imaging was made if the dogs had, besides the abovementioned parameters, a history of at least 6 months of normal interictal period and unremarkable interictal neurological examinations.

Dogs were included in the study if they had a diagnosis of IE and did not achieve a decrease in seizure frequency ≥ 50% despite a treatment of at least four months with a combination of, at least, two or more AEDs at adequate dose. During the treatment, AEDs adequate dosages were guaranteed by serum concentrations of phenobarbital (PB) and bromide (Br) ranging between 20-35 μg/mL and 1000-2000 μg/mL, respectively. For dogs treated using levetiracetam or zonisamide, a minimum dosage of 20 mg/kg q8h and of 7 mg/kg q12h, respectively, was required.

Dogs were excluded from the study in case of MRI or CT abnormal results, seizures starting before six months or after six years of age and in case of incompletely recorded data.

Dogs included in the study were considered affected by RIE and, for practical purposes, were named as “RIE-dogs”.

Clinical data were collected on the basis of the medical records and/or using a standardized owner’s questionnaire and/or contacting by phone the referring veterinarians for additional information.

According to the definition reported in literature, seizures were classified as focal, generalized and focal with secondary generalization (De Risio, 2014; Berendt et al., 2015). (Thomas, 2010). A single episode within 24 hours was recorded as a *single seizure.* More than one seizure occurring within a 24 hours period with full recovery of consciousness between seizures were recorded as *cluster seizures*. A single seizure lasting more than 5 minutes, or two or more epileptic seizures without complete recovery between each episode were recorded as *status epilepticus* (Patterson, 2014).

For each dog, the data recorded included:

- signalment;

- age at first neurological examination;
- age at the time of the first seizure;
- duration of the disorder (defined as the time elapsed from the first seizure to the last follow up);
- number of seizures prior to treatment with AEDs;
- number of seizures after each medication;
- type of seizures (focal, generalized, focal with secondary generalization);
- density of seizures (single, cluster or status epilepticus);
- type of AED administered (including serum levels if dogs were treated with PB or Br);
- adverse effects.

From the establishment providing the major number of refractory cases, in order to assess the clinical risk factors associated with RIE, 94 dogs affected by IE responsive to AED were selected and used as control group (non-RIE dogs). Among these, 22 dogs were randomly choose for the ABCB1 genotyping.

In this population, the diagnosis of IE was made according to the same inclusion criteria used for the RIE dogs.

3.2.1 ABCB1 GENOTYPING

A blood sample in EDTA tube was collected from RIE dogs for ABCB1 genotyping, and from the group of non-RIE dogs. The gDNA was purified using a silica-based column method (NucleoSpin® Tissue gMacherey-Nagel) following manufacturer instructions. The locus of the single nucleotide variation (c.-6-180T>G) (Alves et al., 2011) was PCR amplified using the Phusion Hot Start II DNA Polymerase (ThermoFisher Scientific) and the following forward 5'AGCGCCCAGCTCGGTTTTCA 3' and reverse 5'TTCTCTGCACTCCCCTTACGGCCT primers3'.The PCR mixture for PCR assays included 5 µl 5X Phusion HF Buffer detergent-free, 2.0 mM magnesium chloride, 500 nM each of forward and reverse primers, 200 µM each of dNTPs, 0.5U of Phusion Hot start II Polymerase and 2 µl of template brought up to 25 µl with molecular biology grade water. Each PCR run included negative controls represented by molecular biology grade water. The PCRs were carried out using an EP-gradient S thermalcycler (Eppendorf, Milan,

Italy). The amplification protocol included an initial denaturation at 98°C for 30 s followed by 40 cycles at 98°C for 10 s, at 62°C for 15 s and at 72°C for 15 s, and a final extension step at 72°C for 1 minutes. The PCR products were evaluated after electrophoresis on 1.5% agarose, purified using ExoSAP-IT® PCR Product Clean-Up kit and direct sequenced using the Big-Dye terminator chemistry, additionally purified with Centri-Sep columns (Life Technologies, Monza, Italy) and electrophoresed on an ABI Prism 310 automated sequencer and an ABi.

3.2.2 STATISTICAL METHODS

Data were entered into Excel (Microsoft) and analyzed using a commercially available software (Medcalc software, www.medcalc.net). Standard descriptive analysis was used to describe the population affected by RIE (RIE dogs) and the control group (non-RIE dogs).

Data from the "RIE dogs" group and "non-RIE dogs" group were statistically analyzed to detect risk factors associated with AED refractoriness. The investigated continuous variables were categorized according to: gender (male vs female), reproductive status (neutered vs intact), body weight (> 20 Kg vs < 20 kg) age at seizure onset (>12 month,12-24 month; 25-36 month; >36 month) and seizure type (cluster vs single seizure/generalized vs focal seizure).

Univarite analysis was carried out using Chi-square test both to evaluate the association between the clinical variables and RIE and the allelic state of ABCB1genotype (c.-6-180T>G) and RIE. The Odd ratio was used to assess the association between the allelic state of ABCB1genotype and RIE.

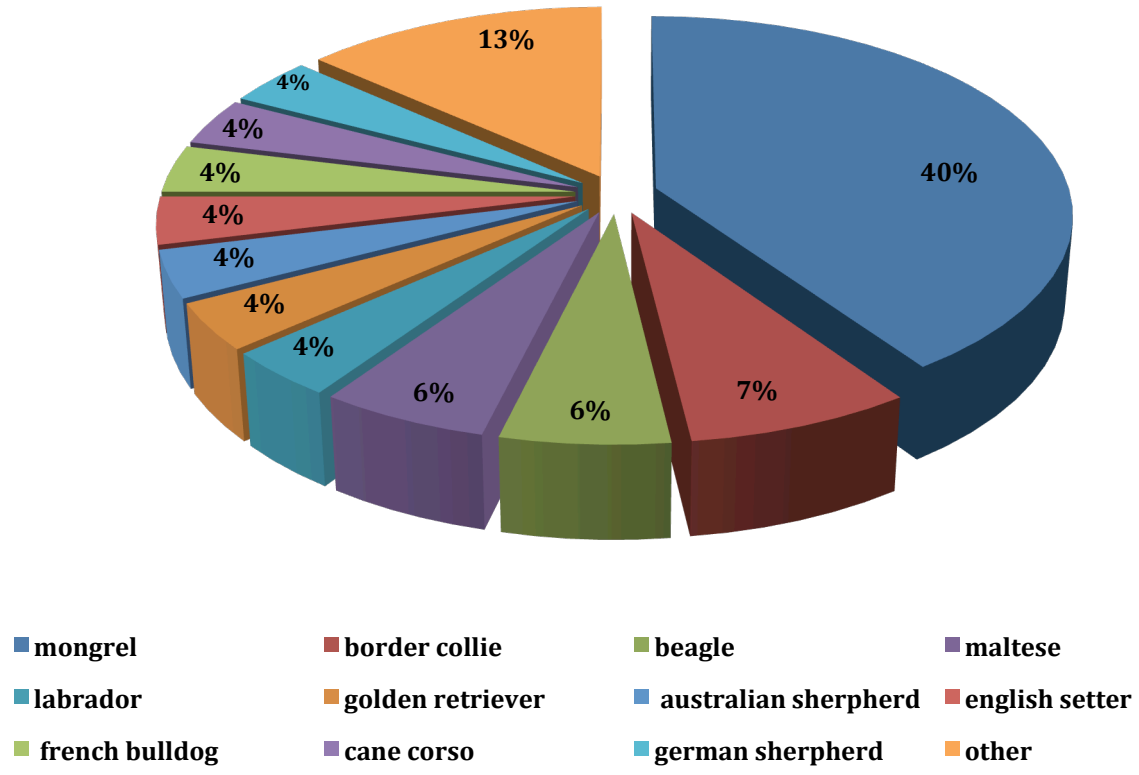
$P \leq 0,05$ was considered statistically significant.

3.3 RESULTS

The clinical records of idiopathic epileptic dogs referred to the six veterinary centers included in the study were retrospectively reviewed. 52 dogs ("RIE dogs" group) fulfilled the inclusion criteria and were enrolled in the study.

"RIE dogs" group

In "RIE dogs" group, a variety of **breeds** was represented including: 21 Mongrel, (40%), 4 Border Collie (7%), 3 Beagle (6%), 3 Maltese (6%), 2 Labrador (4%), 2 Golden Retriever (4%), 2 Australian Shepherd (4%), 2 English Setter (4%), 2 French Bulldog (4%), 2 German Shepherd (4%) and 2 Cane Corso (4%). The remaining population was composed by one dog for each of the following breeds: Boxer; American Staffordshire, Bolognese; Pinscher; Flat Coated Retriever, Dobermann and Jack Russel Terrier.



Thirty-three dogs were **males** (28 intact, 5 neutered) and 19 **females** (12 intact, 7 spayed). (Table1)

The **median weight** was 19,5 kg (IQR:15,5).

The **median age at presentation** to the referral centers was 24 months (IQR:18).

The **median age at onset of seizures** was 24 months (IQR: 24,5).

The **median duration of the disorder** was 20.5 months (IQR:36).

The **median monthly seizure frequency** before medication was 2 (IQR: 3), after the first antiepileptic drug was 3,5 per month (IQR:2,1), after the second 3,75 per month (IQR:1,1).

The most common **seizure type** was tonic-clonic seizure affecting 46 dogs (88%), the remaining 6 dogs (12%) had focal seizures with secondary tonic-clonic generalisation. (Table1). Cluster seizures were present in 29 dogs (55%) including 17 dogs (33%), which experienced status epilepticus.

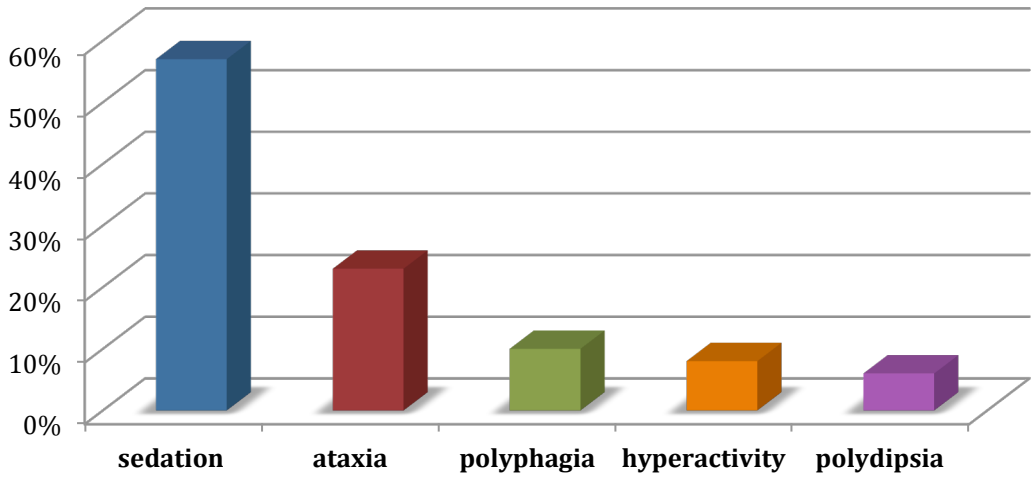
Brain imaging was performed in 37 dogs (71%). MRI and CT, performed respectively in 34 and 3 dogs, were normal in all cases.

The most common used combination of **AEDs** were phenobarbital and bromide (N=41; 79%), phenobarbital and levetiracetam (N=7; 13%), followed by levetiracetam and bromide (N=2; 4%) and phenobarbital and zonisamide (N=2; 4%).

The **median phenobarbital serum concentration** was 25,9 mg/dl (IQR: 6,9).

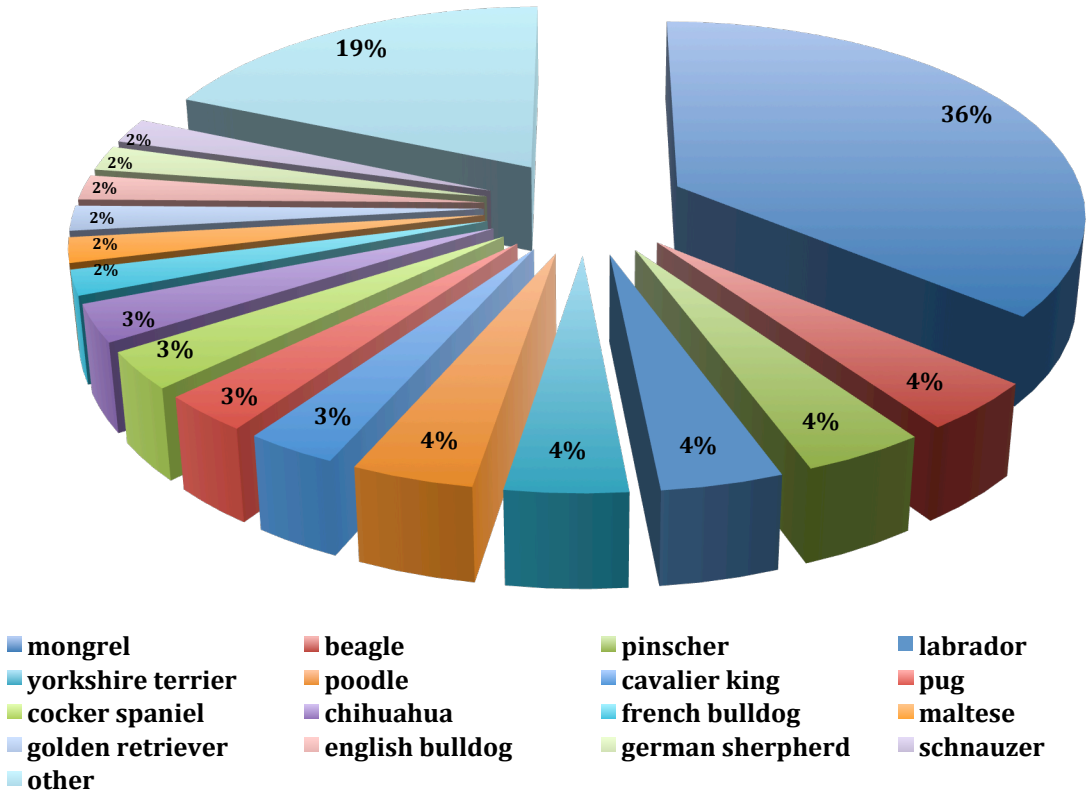
The **median bromide potassium serum concentration** was 1658 µg/ml (IQR: 536) (Table 1). In 25 dogs (49%) a third AED was used to control seizure frequency.

Adverse effects were present in 26 dogs (50%), the most common being sedation (N=14; 57%), ataxia (N=6;23%), polyphagia (N=3;10%) hyperactivity (N=2;8%) and polydipsia (N=1; 6%).



"Non-RIE dogs" group

The control group of non-RIE dogs consisted of 94 dogs, including: 33 Mongrel (35%), 4 Beagle (4%), 4 Pinscher (4%), 4 Labrador Retriever (4%), 4 Yorkshire Terrier (4%), 4 Poodle (4%), 3 Cavalier King Charles Spaniel (3%), 3 Pug (3%), 3 Cocker Spaniel (3%), 3 Chihuahua (3%), 2 French Bulldog (2%), 2 Maltese (2%), 2 Golden Retriever (2%), 2 English Bulldog (2%), 2 German Shepherd (2%), 2 Schnauzer (2%). Seventeen different breeds composed the remaining population. Fifty-six dogs were **males** (52 intact, 4 neutered) and 38 **females** (21 intact, 17 spayed). The **median weight** was 12,7 kg (IQR:18,35). The **median age at presentation** to the referral centers was 52 months (IQR:44,5). The **median age at onset of seizures** was 36 months (IQR: 39,2). Seventy-five dogs (80%) presented tonic-clonic seizures, nineteen (20%) dogs had focal seizures with secondary tonic-clonic generalisation. Eighty- one (86%) presented single seizure and 13 (14%) cluster seizures. Only two dogs experienced a status epilepticus. Eighty-six dogs were treated with one **AED** (84 with phenobarbital, 2 with bromide). The remaining population (8 dogs) were successfully treated with two AEDs (phenobarbital and bromide). The **median phenobarbital serum concentration** was 20,3 mg/dl (IQR: 8,5). The **median bromide potassium serum concentration** was 1469 μg/ml (IQR: 905).



		RIE-dogs	Non RIE dogs
Gender	**Males, intact**	28	52
	Males, neutered	5	4
	Females, intact	12	21
	Females, neutered	7	17
Weight		M=19,5 IQR: 15,5	M=12,7 IQR: 18,35
Age	**at the onset seizure**	M=24 IQR: 24,5	M=36 IQR: 39,2
	at the presentation to referral hospital	M=24 IQR: 18	M=52 IQR: 44,5
Serum AEDs Concentration	**Phenobarbital**	M= 25,6 mg/dl IQR= 6,9	M=20,3 mg/dl IQR: 8,5
	Bromide	M=1658 IQR= 536	M=1469 µg/ml IQR: 905

Table 1 clinical data of RIE and Non RIE dogs.

3.3.1 GENOTYPE RESULTS

From the RIE dogs group, 44 dogs were genotyped to identify ABCB1 mutation (c.-6- 180T >G) previously associated with phenobarbital resistance in BCs. In 8 cases the collected sample was inadequate and the genotyping was not carried out. The mutation was found in 20 dogs (45%), including 13 dogs (29%) in homozygous state and 7 dogs (16%) in heterozygous state. The wild-type genotype was found in 24 dogs (54%). Noteworthy, the ABCB1 mutation occurred in various breeds (Table 4). It was in homozygous state (GG) in: two Border Collie, two Maltese, one Pinscher, one Mongrel, one Labrador, one French Bulldog, one English Setter, one Bolognese, one Beagle, one Australian Shepherd and one American Staffordshire. It was in heterozygous state (TG) in: three Mongrels, one Maltese, one German shepherd, one French bulldog and one Beagle.
From the non-RIE dogs group, 22 dogs were genotyped to identify ABCB1 mutation (c.-6- 180T >G). The ABCB1 mutation was also highly prevalent in the non-RIE group (N=16; 73,7%), including N=5; 31,6% homozygous mutated dogs and N=7; 42,1% heterozygous dogs.

	TG	**GG**
Mongreal	3	1
Pure breed		
Border Collie	0	2
Maltese	1	2
Pinscher	0	1
Labrador	0	1
French Bulldog	1	1
English Setter	0	1
Bolognese	0	1
Beagle	1	1
Australian Sheperd	0	1
German Sheperd	1	0
American Staffordshire	0	1

Table 2 Distribution of ABCB1(c.-6- 180T >G) mutation among mongrel and pure breed **GG**= Mutation in homozygous state , **TG**= Mutation in heterozygous state.

3.3.2 STATISTICAL RESULTS

When compared to the group of non-RIE dogs, Border Collies and Cane Corso were significantly (P<0,001) at higher risk to develop RE.

"Weight > 20 Kg" (Relative Risk [RR]: 1,9; CI 95% 1.1 to 3.2, P=0,01) and "experience of first seizure between 12-24 months" were significantly (RR=2,77, 95%CI= 1.6687 to 4.6008, P= 0,0001,) associated with RIE.

A significant association was found between cluster seizures and RIE (RR= 4,8925, 95%CI= 2.9993 to 7.9808, P < 0,0001) (Table 3).

No significant difference was found between male and female dogs.

The presence of the c.-6- 180T (wild type) was significantly associated with an increased risk of RIE (Odd ratio 3.2, C.I. 95% 1.3 - 7.9; p =0.0134). Conversely, the presence of c.-6-180G allele in homozygous or heterozygous state had a protective effect on the development of RIE (Odd ratio 0.15, C.I. 95% 0.0 – 0.42; p < 0.001).

	RR	**95% CI**	***P***	***z***
Breed				
Border Collie	9,5	7.2 -12.3	<0,0001	16,4
Cane Corso	9,1	7.0- 11.8	<0,0001	16,5
Other breeds				
Weight				
> 20 kg	1,9	1.1 - 3.2	0.01	2,5
< 20 kg				
Seizure type				
cluster	4,9	2.9 - 7.9	<0,0001	6,4
single				
Age onset seizure				
< 12 m^{th}				
12-24 m^{th}	2,7	1.6 - 4.6	0,0001	3,9
25-36 m^{th}				
> 36 m^{th}				

Table 3 Association between clinical variable and Refractory idiopathic epilepsy.

3.4 DISCUSSION

Despite the advances in the treatment of IE, still a relevant percentage of dogs remains poorly controlled (Volk, 2014) and pharmacoresistant canine IE is considered one of the most frustrating condition for pets, owners and neurologists (Chang et al., 2006; Wessmann et al., 2012). In human and veterinary medicine, great effort is spent in trying to elucidate the mechanisms underlying responsiveness and refractoriness to AEDs treatment (Remy and Beck, 2006; Schmidt and Löscher, 2005; Muñana et al., 2012; Alves et al., 2011).

In recent years, attention has been focused on the attempt to identify risk factors to predict the outcome of the disease (Packer et al., 2014; Kwan and Brodie; 2000, Voll et al., 2015; Gomez-Ibanez et al., 2017). When compared to previous papers, the results of this retrospective study provide some information on the clinical and genetic risk factors associated to RIE from a different perspective. In previous studies, risk factors for refractoriness to AEDs treatment were extrapolated from data pertaining to a whole population of idiopathic epileptic dogs (Packer et al., 2014; Arrol et al., 2012). In these studies, risk factors for refractoriness were investigated in epileptic dogs indistinctly treated with one or more AEDs without the precise assessment of drug therapeutic thresholds (Packer et al., 2014; Arrol et al., 2012). In the present study, data from a population of RIE dogs were statistically compared with a control group of AED-responsive dogs. To the authors' knowledge, this is the first investigation on clinical and genetic factors potentially associated to refractoriness focused on a group of dogs affected by RIE selected according to well-defined and strict inclusion criteria.

The present study confirmed that clinical risk factors for RIE include the early onset of seizures and the experience of cluster seizure and identified a higher risk to develop RIE in the Cane Corso and in the Border Collie breed. Furthermore, the study confirmed the presence of the c.-6-180T>G polymorphism in several breeds and failed to identify any association with RIE.

As previously reported, Border Collies suffer from a severe epilepsy course (Hulsmeyer et al., 2010; Arrol et al., 2012). In a German study published in 2010, 71% of BCs treated adequately with ≥ 2 AED had a poor response (≥1 seizure day/month) and the 94% of the study population experienced at least one episode of cluster seizure (Hulsmeyer et al., 2010). These data were confirmed by a recent canine epilepsy study showing that BC is the least likely breed to achieve remission, with a clinical course mostly characterized by cluster seizure (Packer et al., 2014).

Besides BCs, the Cane Corso was found significantly at risk to develop RIE. In the current veterinary literature, there is no specific information about IE in molossoid breeds. A recent report

on IE in the Cane Corso and Dog de Bordeaux, presented at the 28th ESVN-ECVN Annual Symposium depicted a very aggressive epileptic phenotype and a severe course characterized by cluster seizures in 91% of the dogs and by the failure in decreasing seizure frequency in 54% of the population treated with a combination of AEDs (Escriou et al., 2015).

Body weight was identified in the present study as another factor associated with refractoriness. Dogs weighting more than 20 kg were significantly (*P*= 0,01) at higher risk to develop RIE. Previous studies reported that large-breed dogs often suffer from poorly controlled cluster seizures and/or status epilepticus (Knowles, 1998).

Despite a reported male prevalence in the canine epileptic population (Srenk et al., 1994; Kathmann et al., 1999; Patterson et al., 2005; Casal et al., 2006; Gullov et al., 2011; Weisset al., 2012; De Risio et al., 2015), confirmed also in the present study, a significant predisposition of male dogs for RIE was not identified.

Among the group of refractory dogs. the most common seizure type was generalized tonic-clonic seizure. However, the seizure type did not represent a risk factor. Our results confirmed that the experience of cluster seizures was significantly associated with RIE. Human patients affected by cluster seizures are at a higher risk of experiencing worse seizure control and had an increased mortality rate (Haut et al., 2005; Sillanpaa and Schdmit, 2008). In a retrospective study on idiopathic epileptic dogs, the presence of cluster seizures influenced negatively the likelihood of remission (Packer et al., 2014). Our results are aligned with those human and veterinary reports showing that seizure density, considered as the temporal pattern of seizure activity, is a risk factor for the development of AED refractoriness more influential than seizure frequency (Packer et al., 2014; Sillanpää and Schdmit, 2008; Sillanpää, 1993).

Our study showed that higher relative risk to develop RIE was present in those dogs whose onset of seizure occurred between 12-24 month. Several reports on the overall IE canine population outline that dogs experiencing seizures at younger age have a worse outcome than older dogs (Volk, 2014; Packer et al., 2014). In Border collies, seizure onset before the age of 2 years has been shown to significantly decrease survival time (Hulsmeyer et al., 2010). The same finding was reported in a study on Labrador retrievers in which dogs achieving a seizure-free state or an improvement in their seizure frequency, strength and/or duration had a significantly higher age at onset than uncontrolled dogs (Heynold et al., 1997).

In human medicine, seizure onset before the age of 12 months was considered a poor prognostic factor (Mohanraj and Brodie, 2013). Conversely, the single study in veterinary medicine on canine juvenile epilepsy found that younger age at the onset (less than one year) had no influence on survival outcome compared to other studies (Arrol et al., 2012).

Studies on human patients with epilepsy indicate that an early response to drug therapy confers a favorable prognosis (Kwan and Brodie, 2000; Sillanpää, 1993). Patients having a poor response to the first antiepileptic drug had low probability to become seizure-free while taking two drugs. (Kwan and Brodie, 2000). Arrol and coll., in their study on juvenile epilepsy, found that the use of one AED before investigation was associated with a negative outcome, whereas receiving no AED medications before referral was associated with a longer survival (Arrol et al., 2012). In accordance to these findings, our canine population showed a progressive increase in seizure number regardless of the therapy, supporting the hypothesis that some dogs have RIE at the outset rather than developing it over time. Undoubtedly, further studies are necessary to confirm these preliminary findings.

Median serum levels of PB and Bromide were not as higher as probably expected. This may be due to the development of adverse effects preventing further increase in the dosage of the AEDs.

The **evaluation of *ABCB1*** (c.-6-180T>G) SNV frequency in this multi-breed canine population showed the presence of the polymorphism in several breeds but only in 45% of the group of RIE dogs. The potential role of ABCB1 (c.-6-180T>G) polymorphism in RIE dogs was previously evaluated in a study on idiopathic epilepsy in BCs (Alves et al., 2011). Authors found that the single nucleotide substitution (c.-6- 180T>G), identified in a noncoding promoter region of the ABCB1 gene was associated with drug responsiveness in BCs and speculated that it might indicate that regulatory mutations affecting the expression level of ABCB1 could exist, possibly influencing the reaction of a dog to AEDs (Alves et al., 2011). The mutation is located at intron 1 near the 5-end of the gene, where the most important promoter elements are located.

It was hypothesized that this promoter polymorphism might be related to an up-regulation of the gene resulting in an overexpression of P-gp in the brain (Alves et al., 2011). The theory that an overexpression of efflux transport could be associated with RE, was supported by several animal models and human studies (Sisodiya et al., 2002; Loscher and Potschka, 2005).

In BCs, the significance of this finding was recently cast to doubt by a Japanese study that detected the same SNV in a normal BC population, with a frequency of 9.8 % in homozygous state and 30,3% in heterozygous state (Mizukami et al., 2013). In the present study, gene mutation (c.-6-180T >G) was present in 47% of the RIE dogs, while the 53% presented the wild type T/T. Our findings in the multi-breed group of RIE dogs did not support a role of this polymorphism in refractoriness since its presence neither in homozygous and in heterozygous state was associated with an increased risk. On the contrary, a significant reduced risk was shown for the presence of the polymorphism. These data weaken the hypothesis that a strong and direct detrimental effect of this variation *per se* could occurs. Clearly, only in BCs, the intron 1 variation could interact with other

variations causing the refractoriness or, alternatively, is in linkage disequilibrium with another mutation causing the refractoriness which has not be found out by the sequencing of the ABCB1 exons and flanking sequences of (Alves et al., 2011). Being in different genomic landscape of other breeds, this effect is not evident. Consequently, the c.-6-180T>G allele did not explain the refractoriness, also the half of dogs carrying it either in heterozygous or homozygous state

The results about studies evaluating ABCB1 heterogeneity in epileptic people are conflicting. Meta-analysis failed to identify an association between genotype and treatment response, so its significance remains uncertain also in people. (Haerian et al., 2010, Sun et al., 2014). Further studies are needed to clarify the role of this mutation in the development of RIE in dogs.

The main limitations of this study are those associated with a retrospective/prospective investigation and the use of a referral population. The dogs were selected from clinical referral centers specialized in veterinary neurology thus they may not be representative of the general canine idiopathic epileptic population. Another limitation is the lack in all RIE dogs of advanced diagnostic imaging to exclude symptomatic epilepsy. However, the strict inclusion criteria made very unlikely the enrolment of dogs with a structural brain lesion. The data collection occurred by questionnaires distributed to the owners and results largely relied on retrospective data evaluation and owners description, allowing possible misinterpretation and an overestimation of seizure. Another limitation cobnsists in the variability of the AED treatment protocols, due the retrospective multicentic nature of the study. Finally, the control group (non RIE dogs) reflected the general IE referral population of a single establishment and not the whole population of all the centers involved in the study.

4.1 CONCLUSIONS

The present study confirmed that an early onset of seizure and the experience of cluster seizure are risk factors of RIE, and identified a greater risk to develop RIE for the Border Collie and Cane Corso breeds. Furthermore, our study showed the presence of the c.-6-180T>G polymorphism in different breeds of both the RIE and non-RIE groups of dogs, failing to identify any association with RIE.

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