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Original Article

ABCB1 c.-6-180T>G polymorphism and clinical risk factors in a multi-breed cohort of dogs with refractory idiopathic epilepsy

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Highlights

- *ABCB1* c.-6-180T>G polymorphism has been found in several canine breeds with idiopathic epilepsy, besides Border collies
- The *ABCB1* c.-6-180G allele was not significantly correlated with refractory idiopathic epilepsy in a multi-breed population
- The effect of the *ABCB1* c.-6-180T>G polymorphism is likely modulated by genetic backgrounds of the various breeds
- Cluster seizures represent the main clinical risk factor for refractoriness

Abstract

Epilepsy is the most common chronic neurological disorder in dogs. Approximately 20-30% of dogs do not achieve satisfactory seizure control with two or more anti-epileptic drugs at appropriate dosages. This condition, defined as refractory epilepsy, is a multifactorial condition involving both acquired and genetic factors. The P glycoprotein might play and important role in the pathophysiological mechanism and it is encoded by the *ABCB1* gene. An association between a single nucleotide variation of the *ABCB1* gene (c.-6-180T>G) and phenobarbital resistance has previously been reported in a Border collie population with idiopathic epilepsy. To date, the presence and relevance of this polymorphism has not been assessed in other breeds. A multicentre retrospective, case-control study was conducted to investigate associations between *ABCB1* c.-6-180T>G, clinical variables, and refractoriness in a multi-breed population of dogs with refractory idiopathic epilepsy. A secondary aim was to evaluate the possible involvement of the *ABCB1* c.-6-180T>G single nucleotide variation this population.

Fifty-two refractory and 50 responsive dogs with idiopathic epilepsy were enrolled. Of these, 45 refractory and 50 responsive (control) dogs were genotyped. The G allele was

found in several breeds, but there was no evidence of association with refractoriness (*P*=0.69). The uncertain role of the c.-6-180T>G variation was further suggested by an association between the T/T genotype with both refractoriness and responsiveness in different breeds. Furthermore, high seizure density (cluster seizure) was the main clinical risk factor for refractory idiopathic epilepsy (*P*=0.003).

Keywords: ABCB1; Anti-epileptic drugs; Canine; Epilepsy; Refractory idiopathic epilepsy; Risk factors

Introduction

Idiopathic epilepsy (IE) is considered the most common chronic neurological disease in dogs, with an estimated prevalence varying from 0.5 to 5% in the general canine population (Podell 1995; Ekenstedt and Oberbaur, 2013; Hülsmeyer et al., 2015). The treatment of canine IE is symptomatic and consists of the administration of anti-epileptic drugs (AEDs) aimed at decreasing the frequency and severity of the seizures (Bhatti et al., 2015). Approximately 75-85% of IE dogs continue to have seizures, and 20-30% of this population do not achieve satisfactory seizure control with two or more AEDs at appropriate dosages (Berendt et al., 2002; Arrol et al., 2012). This condition has been defined as Refractory Epilepsy (RE; Schwartz-Porsche et al., 1985; Podel and Fenner, 1993; Trapanier et al., 1998; Muñana, 2013).

Refractory Idiopathic Epilepsy (RIE) is a multifactorial condition involving acquired and genetic factors (Kwan and Brodie, 2002; Volk, 2014). In humans, high seizure frequency before treatment and inadequate response to initial AED treatment have been identified as clinical risk factors of refractoriness (Kwan and Brodie, 2000; Mohanraj and Brodie 2013). In the dog, cluster seizures are the main risk factor for AED unresponsiveness (Packer et al., 2014). The hypothesis of a genetic influence of refractoriness was supported by reports of high percentages of a particularly severe clinical course and refractoriness in specific breeds, such as Border collies (BCs; Hülsmeyer et al., 2010; Arrol et al., 2012; Packer et al., 2014), Australian Shepherds (Weissl et al., 2012) and Italian Spinones (De Risio et al., 2015).

The exact pathophysiological mechanisms of refractoriness are still poorly understood (Stepien et al., 2012). In recent years, specific attention has been drawn to the role of the ATP-binding cassette subfamily B member 1 protein (*ABCB1*, formerly known as multidrug

resistance protein 1 or P-glycoprotein), encoded by the *ABCB1* gene (Kwan and Brodie, 2005). The P-glycoprotein is an ATP-dependent transmembrane protein expressed physiologically on the luminal side of the endothelial cells of the blood brain barrier. It has a protective physiological function by excreting potentially toxic xenobiotics, including AEDs. An overexpression of these efflux transporters, due to mutation in the coding sequence or in the regulatory regions of the *ABCB1* gene, may inhibit AED penetration in epileptic foci, resulting in reduced efficacy of antiepileptic treatment (Kwan and Brodie, 2005; West and Mealey 2007).

In human medicine, several studies have been conducted to evaluate the association between ABCB1 polymorphism and the resistance to AEDs, showing discordant findings (Chouchi et al., 2017). In dogs, a 2011 study by Alves et al. reported that the *ABCB1* c.-6-180T>G single nucleotide variation (SNV) was associated with phenobarbital resistance in a population of BCs with IE (Alves et al., 2011).

To date, the presence and relevance of this SNV have not been ascertained in other breeds. The early identification of refractoriness and the recognition of the genetic variations underlying drug unresponsiveness may have relevant clinical and therapeutic implications in improving the outcome and quality of life of dogs with RIE.

The aims of this study were to assess the association between *ABCB1* SNV, clinical variables, and refractoriness, using multiple logistic regression modelling in a multi-breed population of dogs with RIE; evaluate the frequency of the *ABCB1* c.-6-180T>G SNV in this multi-breed population, and provide a detailed description of the clinical presentation.

Materials and methods

In the present retrospective case-control study, medical records of dogs with IE presented between January 2010 and December 2014 to six neurological referral centres were reviewed; blood samples were collected for genotyping. The study was carried out according to the ethical rules and with the approval of the Ethical Committee of the Bologna University (Approval number: ID 815/Prot 3886; Approval date: 21 July 2017).

The diagnosis of IE was based upon the dogs satisfying all of the following criteria: a history of two or more unprovoked epileptic seizures occurring at least 24 h apart; onset of seizures between 6 months and 6 years of age; normal interictal neurological examination; and unremarkable blood results (including complete blood cell count, clinical chemistry panel including, in most cases, fasting bile acid and/or ammonia). For those dogs which underwent advanced diagnostic imaging, MRI (*n*= 44) or CT (*n*= 3) of the brain had to be normal. For those dogs that did not undergo advanced diagnostic imaging, a 6-month follow-up with normal interictal periods and unremarkable interictal neurological examinations were necessary to be included in the study.

Dogs were defined as having RIE when they failed to achieve a decrease in seizure frequency ≥ 50% despite a treatment of a minimum of 6 months with a combination of an adequate dosage of at least two AEDs. During the treatment, adequate dosages of AED were assessed by serum concentrations of phenobarbital (PB) and bromide (Br), with ranges from 20-35 μg/mL and 1,000-2,000 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. For practical purposes, this group was called ‘dogs with RIE’.

Control dogs 'without RIE' were selected using identical inclusion criteria for dogs with RIE, except for the response to AEDs treatment. In these dogs, AEDs produced a decrease in seizure frequency > 50%. No matching criteria were used for selecting control-group dogs to avoid bias in statistical analysis.

Clinical data were collected based on the medical records and/or using a standardised owner's questionnaire and/or contacting the referring veterinarians by phone for additional information. For each dog, the data recorded included: signalment, age at first neurological examination, age at the time of the first seizure, number of seizures prior to treatment with AEDs, number of seizures after each type of medication, type of seizures (focal or generalised), density of seizures (single seizures, cluster seizures [CS] or status epilepticus [SE]), type of AED administered (including serum levels if dogs were treated with PB or Br) and adverse effects. In the case of CS, each single seizure of the event was counted as one seizure.

ABCB1 genotyping

The genomic DNA was purified using a silica-based column method (NucleoSpin® Tissue gMacherey-Nagel) from K_3 EDTA anticoagulated blood, following the manufacturer's instructions. Among different methods of genotyping evaluated, the direct sanger-sequencing method was used for genotyping the SNV *ABCB1* c.-6-180T>G (Alves et al., 2011). Because this SNV locus lies within three 10-nucleotide repeats, the use of most alternative typing methods, i.e. dual-labeled fluorescent probes or restriction length fragment polymorphism, is precluded. Validation of the Sanger-sequencing genotyping assay was confirmed in previous work, using two different primer pairs to genotype unknown samples; this approach was used to exclude drop-out effect (Turba et al., 2017). After the validation study, a single primer pair

was established and used here for the genotyping assay. The locus of interest was amplified via PCR, using Phusion Hot Start II DNA Polymerase (ThermoFisher Scientific) and forward 5'AGCGCCCAGCTCGGTTTTCA 3' and reverse 5'TTCTCTGCACTCCCCTTACGGCCT 3'primers. Each PCR run included negative controls, where DNA was replaced by molecular biology grade water. 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) and electrophoresed on an ABI Prism 310 automated sequencer and an ABI (Fig.1).

Statistical methods

Standard descriptive analysis was used to describe the population with RIE and without RIE, using commercially available software [1]. Data were presented as median with 25th and 75th percentiles.

Univariate analysis modelling was used to evaluate the associations between each variable and the classification of dogs as having RIE, together with odds ratios (ORs) and 95% confidence intervals (CIs). Logistic regression analysis was used to determine the ORs and the p value. In addition, Fisher's Exact test was used for assessing the breed factor with the Cornfield approximation to calculate the ORs for those breeds represented only in one group (dogs with RIE or without RIE). For statistical purposes, breeds represented with n = <3 were grouped as 'other breeds'.

[1] See: Medcalc software. www.medcalc.org (Accessed 10 September 2019).

The variables were then modelled using multiple regression analysis included in the STATA v11 package (Stata), creating several models. Continuous variables included bodyweight, age at seizure onset, and age at first neurological examination. Age at seizure onset, and age at first neurological examination were also categorised into four groups: < 12 months; ≥ 12 and < 24 months; ≥ 24 and < 36 months; ≥ 36 months. Categorical (factor) variables included sex (intact female, spayed female, intact male and neutered male), seizure type (cluster and single seizures; generalised and focal seizures) and breed.

The *ABCB1* c.-6-180 genotype was investigated as a predictor variable. All hypotheses were made on the inheritance pattern of the c.-6-180T>G polymorphism, and either a dominant effect (T/T genotype vs.T/G and G/G genotypes), or a recessive effect (T/T and T/G vs.G/G genotypes) or an incomplete dominance (T/T vs.T/G vs.G/G) of the G allele were evaluated. Results were considered statistically significant at $P \leq 0.05$.

Results

Fifty-two dogs with RIE and 50 dogs without RIE were enrolled in the study.

Descriptive data

Mixed-breed dogs were the majority in dogs with RIE (*n* = 21; 40%) as well as in that without RIE (*n*=16; 32%). In dogs with RIE, the pure-breed most represented was the BC (*n* = 4; 8%), while in dogs without RIE, it was the Labrador retriever (*n* = 5; 10%; Table 1). Male dogs were markedly represented in both dogs with RIE (*n* = 33; 73%) and without RIE (*n* = 34; 68 %). The median weight of dogs with RIE (19.5 kg; range, 14.5-30.2 kg) and dogs without RIE (16 kg; range, 10-29 kg) was almost equal. The group of dogs with RIE showed an earlier onset of seizures (24 months; 12-36.7 vs. 36 months; range, 21.2-54 months) and

were presented much earlier at first neurological examination than dogs without RIE (24 months; 18-41.7 vs. 50 months; range, 25.7-71.7 months). In dogs with RIE, the median monthly seizure frequency before medication was two seizures per month (1.5-4/month); after treatment with the first AED, it was 3.5 seizures per month (2-4.2/month), and after combination treatment with two AEDs, it was 3.5 seizures per month (2-5/month). In dogs without RIE, the median monthly seizure frequency before medication was one seizure per month (1-2/month), and after the AED treatment, seizures were less than one per month (0-0.2/month).

In the RIE group, adverse effects were recorded in 25 dogs (48%): sedation (*n* = 14; 56%), ataxia (*n* = 6; 24%), polyphagia (*n* = 5; 20%), hyperactivity (*n* = 4; 16%), polydipsia (*n* = 3; 12%) and pancreatitis (*n* = 2; 8%). In dogs without RIE, adverse effects were present only in seven dogs (14%) and consisted of sedation, polyphagia, polydipsia in three dogs, sedation in two dogs, ataxia in one dog, and sedation and ataxia in one dog. For both groups, full medical data records are reported in Table 2.

Genotyping data

Forty-five dogs with RIE were genotyped to identify *ABCB1* SNV (c.-6- 180T >G; Fig. 2). In the remaining seven cases, the sample collected was inadequate, and genotyping was therefore not carried out. The SNV c.-6-180T>G variant occurred in 19 dogs (42%), including 12 G/G homozygous dogs (27%) and seven heterozygous dogs (16%). The wild-type genotype (T/T) occurred in 26 dogs (58%). It is worth noting that the *ABCB1* SNV occurred in various breeds.

Fifty dogs without RIE were genotyped to identify the *ABCB1* SNV (c.-6- 180T >G). In this group, the frequencies of the genotypes were 36% (*n* = 18), 38% (*n* = 19) and 26% (*n* = 13) for the TT, GT and GG genotypes, respectively.

Risk factors

According to the univariate analysis, the clinical factors significantly associated with RIE were as follows: the experience of cluster seizures (OR 12.02; 95% CI, 3.70-39.02; *P* < 0.01) and the occurrence of status epilepticus (OR 22.12; 95% CI 2.77-176.78; *P* < 0.01). With the exception of BCs, (OR 1.22; 95% CI, 1.21 – not determined; *P* = 0.043), no breeds were associated with RIE. Furthermore, there was no significant association with weight, type of seizure (focal vs. generalized), sex, age at onset of seizures, or age at first neurological examination (Table 3).

When categorised according to a dominant effect of the G allele (T/T genotype vs.T/G and G/G genotypes), the genotype TT was significantly associated with RIE (OR 2.43; 95% CI, 1.06 – 5.56; *P* = 0.03). Conversely, there was no association when cases were categorised according to a recessive inheritance pattern of the G allele (T/T and T/G vs. G/G genotypes; *P* = 0.69). When categorised according to an incomplete dominance pattern (T/T vs. T/G vs.G/G), the heterozygote T/G genotype was inversely associated with RIE when compared with the T/T genotype (OR 0.31; 95% CI, 0.11 – 0.86; *P* = 0.02; Table 3).

Subsequently, the factors were entered into a multiple logistic regression analysis for modelling the RIE occurrence. Numerous significant models were obtained, mostly accounting for few predictors consistently reliable in modelling refractoriness. Interestingly, only the experience of cluster seizures remained significant in all models; its contribution to

the modelling was strong, while the presence of status epilepticus as well as the genotype predictors did show a reduction in their relevance with respect to the univariate analysis, showing only a limited/moderate impact on the modelling and not reaching significance in the majority of models.

To investigate the relationship between factors, a further predictor, namely the interaction between breed and genotype, was determined and entered in the multiple regression analysis. After adjusting for the interaction, we demonstrated that the T/T genotype effect was dependent on the breed factor, as it was either associated with RIE or non-RIE status in the subgroup of breeds represented with less than three dogs (T/T and other breed OR 31.81, 95% CI, 1.09- 930.94; $P = 0.045$*)* and Labradors, respectively (T/T and Labradors OR 0.00, 95% CI, 0.0001 – 0.80; $P = 0.042$; Table 4). Indeed, few other predictors, and in particular, sex (male intact vs. male neutered OR 0.01; 95% CI, 0.00 – 1-14; $P = 0.057$), age at first neurological examination (24-36 months vs. > 36 months, OR 59.90, 95% CI, 2.85 – 1259.17; $P < 0.01$), the presence of status epilepticus (OR 172.45, 95% CI, 0.22 – 1.33 x 10^5; $P =0.129$) and cluster seizures (OR 181.28, 95% CI 5.84 – 5630.21; $P= 0.003$), beside the interaction between genotype and breed, conferred the most relevant contributions to the model (Table 4).

Discussion

In recent years, genetic factors influencing the AED response have been investigated only in single-breed populations of idiopathic epileptic dogs (Alves et al., 2011; Muñana et al., 2012; Weissl et al., 2012). Furthermore, only a single paper regarding a large population of dogs, focusing on the identification of clinical risk factors associated with AED responsiveness in canine IE, has been published (Packer et al., 2014). To the authors'

knowledge, this is the first study evaluating potential risk factors associated with refractoriness in a multi-breed population of dogs, integrating genetic and clinical variables.

Our results demonstrated the presence of the G allele (c.-6-180T>G SNV) in several canine breeds, but there was no association between its presence (both in homozygous or heterozygous states) and refractoriness. Interestingly, in our study, the T major allele when homozygous was associated either with increased or decreased Odds of RIE in selected breeds or group of breeds. In other words, our results suggested that the effect of the genotype, if any, can be greatly variable according to the particular genetic background characterising certain breeds represented in this study.

In human medicine, several polymorphisms of the *ABCB1* gene have been investigated, and the C3435T polymorphism (rs1045642) has been suggested as one of the genetic factors underlying drug resistance (Schmidt and Löscher, 2005). Nevertheless, the results of studies evaluating *ABCB1* in epileptic people are conflicting. Some studies have reported that the C/C genotype was more prevalent in drug-resistant epilepsy (Siddiqui et al., 2003; Zimprich et al., 2004), while other studies have reported an association with the T/T genotype (Seo et al., 2006; Kwan et al., 2007).

In dogs, the potential role of the *ABCB1* (c.-6-180T>G) polymorphism has previously been evaluated in two studies on BCs (Alves et al., 2011; Mikazumi et al, 2013). In the first study (Alves et al., 2011), the authors reported an association between a single nucleotide substitution (c.-6- 180T>G) of the *ABCB1* gene and PB unresponsiveness in that breed. The mutation, located in a noncoding region (at intron 1 near the 5-end of the gene), is where the promoter elements are located. It was hypothesised that this promoter polymorphism might

be related to an up-regulation of the gene, resulting in an overexpression of P-glycoprotein in the brain (Alves et al., 2011). Indeed, several animal models and human studies have supported the theory that an overexpression of efflux transport could be associated with RE (Sisodiya et al., 2002; Löscher and Potschka, 2005). The second study (Mikazumi et al., 2013) demonstrated a high prevalence of the G allele (24.9%) in a population of normal BCs and concluded that (in a putative dominant inheritance pattern) more than 40% of the dogs had genotypes (G/G and T/G) potentially predisposing to refractoriness in case of development of IE (Mizukami et al., 2013). Based on our results, BC was the only breed significantly associated with RIE. All four BCs were classified as dogs with RIE and only two of them showed the G/G allele in the homozygous state. The small number of dogs in this breed does not allow drawing of conclusions, but it is interesting to note that 50% of the refractory BCs did not present the G allele.

The different findings in human medicine stimulated several meta-analyses, the majority indicating that no association existed between refractoriness and the *ABCB1* polymorphisms (Haerian et al., 2010; Emich-widera et al., 2014; Sun et al., 2014). However, many showed a significant association in specific ethnic subgroups (Zimprich et al., 2008; Menzler et al., 2014). In human medicine, ethnicity is a factor that may affect the results; an allele may become more common in one ethnic subgroup, but not in another (Haerian et al., 2011; Chouchi et al., 2017). Likewise, in our multi-breed population, the different genetic background of each breed may bias the biological effect of this SNV.

In the present study, according to our statistical modelling, it is unlikely that a strong and direct detrimental effect of this variation may occur *per se*. It is more conceivable that the SNV is in linkage disequilibrium with the actual causative mutation producing the

refractoriness to AEDs, but with different strengths of linkage in the various breeds. Alternatively, it is also possible that the intron 1 variation interacts with modifiers that vary in the different genetic backgrounds.

From a practical viewpoint, our results suggest that the genetic assessment of the *ABCB1* c.6180T>G is not of clinical significance, and also suggest that this polymorphism is not itself causative of refractoriness, but it may play a role in a particular genetic background. Hence, epistatic mechanisms may take place also in dogs to modulate RIE. Additional studies are necessary to clarify the role of this gene in the development of RIE in dogs.

Regarding the clinical risk factors, the occurrence of CS represents the single greatest risk factor for RIE. These results are aligned with those from 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 and is more influential than seizure frequency (Sillanpää and Schmidt, 2008; Packer et al., 2014). Human patients with CS are at higher risk of experiencing lack of seizure control and have an increased mortality rate (Haut et al., 2005; Sillanpää and Schmidt, 2008). In the above-mentioned study on risk factors associated with drug responsiveness in idiopathic epileptic dogs, the presence of CS negatively influenced the likelihood of remission (Packer et al., 2014).

In dogs with RIE, the current study showed a progressive increase in seizure number regardless of the therapy, while dogs without RIE achieved good to excellent seizure control using a single AED. Studies on human patients indicate that an early response to drug therapy was associated with a favourable prognosis (Kwan and Brodie, 2000; Sillanpää, 1993) and that patients having a poor response to the first AED had low probability of

becoming seizure-free while taking two drugs. (Kwan and Brodie, 2000). These findings may suggest that RIE is a clinical condition developing at the outset rather than over time. Further investigation is warranted to confirm this hypothesis in dogs. In accordance with the most recent literature (Erlen et al., 2018), in the current study male dogs were over-represented in both groups. Despite not reaching statistical significance, male dogs showed increasing odds of developing RIE.

In the present study population, the median serum levels of PB and Br were not as high as expected, which is most likely due to the development of adverse effects, preventing an additional increase in the dosage of the AEDs. Indeed, in dogs with RIE, 50% of the population showed adverse effects versus 7% of dogs without RIE. Unfortunately, the retrospective nature of the study prevents a confirmation of our hypothesis.

The major limitation of this study was the small population size studied. The difficulty of collecting a numerically adequate population is one of the most important weaknesses in many veterinary studies. We report results from just 95 genotyped dogs; another study by Alves et al. (2011) examined association between the G allele and refractoriness in BCs, based on a population of 25 dogs. The entire premise that this variant causes AED resistance, is completely unproven. While our study is a relevant next step in the investigation of potential relationships between this variant and AED resistance, larger studies are required to further investigate any links. Furthermore, the lack of pedigree information in purebred dogs in our study did not allow us to evaluate their relatedness; this might have affected our findings particularly in BCs and Labradors. Another limitation is the retrospective study design. Data were collected using both clinical records and, especially for the follow-up data, questionnaires distributed to the owners, allowing possible

misclassification of seizure type. Additionally, advanced diagnostic imaging was not performed in all dogs with RIE to exclude structural epilepsy. However, the strict inclusion criteria made the enrollment of dogs with a structural brain lesion very unlikely. Finally, the inherent variability of AED treatment protocols due to the retrospective multicentric nature of the study could have introduced non-differential bias.

Conclusions

This study demonstrated c.-6-180T>G polymorphism in dogs of different breeds, both with and without RIE and failed to identify any association with refractoriness. These results suggest that this variation is unlikely to be directly responsible for refractoriness, but may play a variable role according to the particular genetic background. Furthermore, in this study, a history of cluster seizures was the main clinical risk factor for the development of RIE.

Conflict of interest statement

The authors of this paper have no financial or personal relationships with other people or organisations that could inappropriately influence or bias the content of the paper.

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Table 1

Breeds represented in the group of dogs with and without refractory idiopathic epilepsy (RIE).

Dogs with RIE	*n*		Dogs without RIE	*n*
Mix-breed	21	(three not genotyped)	Mix-breed	16
Border collie	4		Labrador retriever	5
Maltese dog	3		German shepherd	3
Beagle	3	(one not genotyped)	Poodle	3
Labrador retriever	2		French bulldog	3
Golden retriever	2		Beagle	2
Australian shepherd	2		[a] Schnauzer	2
English setter	2		[a] Chihuahua	2
French bulldog	2		Australian shepherd	1
German shepherd	2	(one not genotyped)	Maltese dog	1
[a] Cane Corso	2	(one not genotyped)	Golden retriever	1
[a] Boxer	1		[a] Cavalier King Charles spaniel	1
[a] American Staffordshire	1		[a] Pug	1
[a] Bolognese	1		[a] English bulldog	1
[a] Pinscher	1		[a] Anatolia shepherd	1
[a] Flat coated retriever	1		[a] Akita Inu	1
[a] Dobermann	1	(not genotyped)	[a] Bloodhound	1
[a] Jack Russell terrier	1		[a] Jack Russell terrier	1
			[a] Dachshund	1
			[a] Great Dane	1
			[a] Pinscher	1
			[a] Spitz	1

[a] Breeds represented with *n* = <3 that for statistical purpose were grouped as 'other breeds'.

Table 2

Clinical data of seizure type, seizure density, and type of treatment in dogs with and without refractory idiopathic epilepsy (RIE).

		Dogs with RIE *n* (%)		Dogs without RIE *n* (%)	
Sex	Males intact	28	(54)	32	(64)
	Males neutered	5	(10)	2	(4)
	Females intact	12	(23)	7	(14)
	Females neutered	7	(13)	9	(18)
Seizure type and density	Tonic-clonic	46	(88)	46	(92)
	Focal	6	(12)	4	(8)
	Cluster	29	(55)	7	(14)
	Status epilepticus	17	(33)	1	(2)
Type of treatment	Pb + Br	38	(73)	Pb 47	(94)
	Pb + Levetiracetam	10	(19)	Br 3	(6)
	Pb + Zonisamide	2	(4)		
	Levetiracetam + Br	2	(4)		
Advance brain imaging	MRI	34	(65)	10	(20)
	CT	3	(6)		
AED serum concentration	Phenobarbital	26.5 µg/mL	(23-28.3)	21.4	(18.6-23.8)
	Bromide	1,640 µg/mL	(1350-1937)	2,033	(1925-2090.5)

Table 3

Results of the univariate analysis.

Independent variables	OR	SE	z	Probability	95% CI
Dominant genotype					
T/T vs. T/G and G/G	2.43	1.02	2.1	0.03	1.06-5.56
Recessive genotype					
T/T and T/G vs. G/G	1.20	0.56	0.39	0.69	0.47-3.01
Genotype					
T/G	0.31	0.16	-2.25	0.02	0.11 – 0.86
G/G vs. T/T	0.54	0.28	-1.20	0.23	0.20 – 1.47
Bodyweight	1.00	0.01	0.33	0.74	0.97-1.03
Age at seizure onset (months; categorical)					
< 12	1.2	0.92	0.24	0.81	0.26-5.39
≥12 and < 24	2.3	1.14	1.76	0.09	0.86-6.11
≥ 24 and <36	0.75	0.46	-0.64	0.64	0.22-2.54
≥ 36					
Age at first neurological examination (months; categorical)					
< 12					
≥12 and < 24	0.83	1.07	-0.14	0.88	0.06-10.55
≥24 and < 36	1.62	2.19	0.36	0.71	0.11-22.98
≥36	0.20	0.26	-1.24	0.21	0.01-2.47
Seizure type					
Focal vs. generalised	1.09	0.70	0.14	0.89	0.30-3.85
Seizure type					
Single vs. cluster	12.02	7.22	4.14	<0.01	3.70-39.01
Status epilepticus					
absent vs. present	22.12	23.46	2.92	<0.01	2.77-176.78

[a] Breed (n < 3 grouped as other breeds)					
Mixed-breed					
Labrador retriever	1.8			0.21	0.50-4.50
Golden retriever	0.43			0.44	0.04-2.87
Beagle	2.66			0.45	0.20-34.19
Australian shepherd	1.33			0.79	0.15-11.35
Border collie	2.66			0.45	0.20-34.19
Maltese	1.22			0.04	1.21 -
French bulldog	4			0.26	0.35-45.09
German shepherd	0.88			0.90	0.12-6.48
Poodle	0.44			0.51	0.03-5.01
	0.00			0.25	– 1.45
Sex					
Neutered female					
Intact female	2.2	1.61	1.07	0.28	0.52-9.29
Intact male	1.2	0.75	0.29	0.77	0.34-4.13
Neutered male	4	4.04	1.37	0.17	0.54-29.06
Sex					
Female					
Male	0.85	0.37	0-.37	0.71	0.36-1.99

SE, standard error; OR, Odds ratio; 95% CI, 95% Confidence intervals

[a] Breeds were analysed using the Fisher's Exact test

Table 4

Multiple logistic regression analysis including genotype and breed interaction. [a]

Independent variables	OR	SE	z	*P*	95% CI	
Sex						
Female neutered	0.44	0.71	-0.51	0.611	0.02	10.47
Female intact	0.90	1.10	-0.08	0.934	0.08	9.71
Male intact vs. male neutered	0.01	0.03	-1.91	0.057	0.00	1.14
Age at first neurological examination						
<12 months	0.11	0.21	-1.12	0.264	0.00	5.41
≥12 <24 months	2.00	1.89	0.74	0.462	0.31	12.72
≥24 <36 months	59.90	93.04	2.63	0.008	2.85	1259.17
vs. ≥36 months						
Seizure type						
Single vs. cluster	181.28	317.79	2.97	0.003	5.84	5630.21
Status epilepticus						
Absent vs. present	172.45	585.03	1.52	0.129	0.22	$1.33x10^5$
Genotype dominant # breed						
genotype T/G and G/G Mixed breeds	0.34	0.44	-0.83	0.409	0.03	4.34
Genotype T/G and G/G Labradors	3.70	6.07	0.80	0.425	0.15	92.25
Genotype T/G and G/G Beagles	0.00	0.01	-1.51	0.131	0.00	6.89
Genotype T/G and G/G Australian shepherds	0.69	1.40	-0.18	0.854	0.01	37.56
Genotype T/G and G/G Border collies	0.65	1.61	-0.17	0.861	0.00	84.17
Genotype T/T Other breeds	31.81	54.80	2.01	0.045	1.09	930.94
Genotype T/T Mixed-breeds	2.64	2.98	0.86	0.388	0.29	24.02
Genotype T/T Labradors	0.00	0.01	-2.03	0.042	0.0001	0.80
vs. genotype T/G and G/G other breeds						

SE, standard error; OR, Odds ratio; 95% CI, 95% Confidence intervals

[a] Log likelihood, -24.39; Pseudo $r^2 = 0.52$; $P < 0.001$

Figure legends

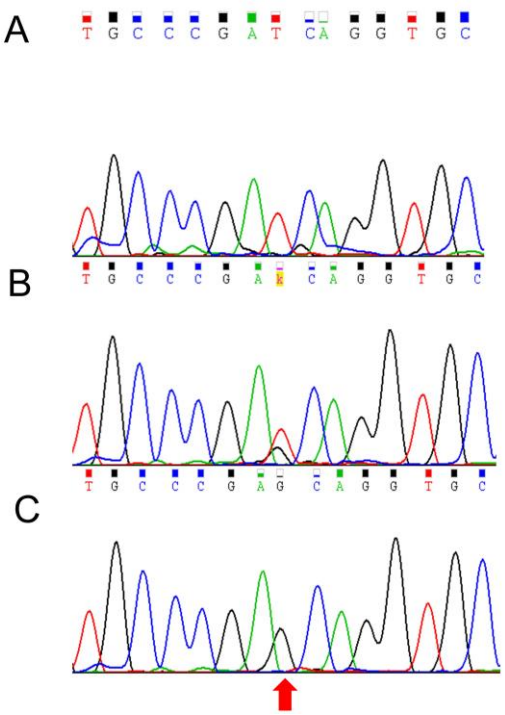

Fig. 1. Representative sequencing chromatograms of the genotyping assay. The c.-6- 180T>G polymorphism of the *ABCB1* gene is indicated by an arrow. A) T/T genotype; B) T/G genotype; and C) G/G genotype are represented.

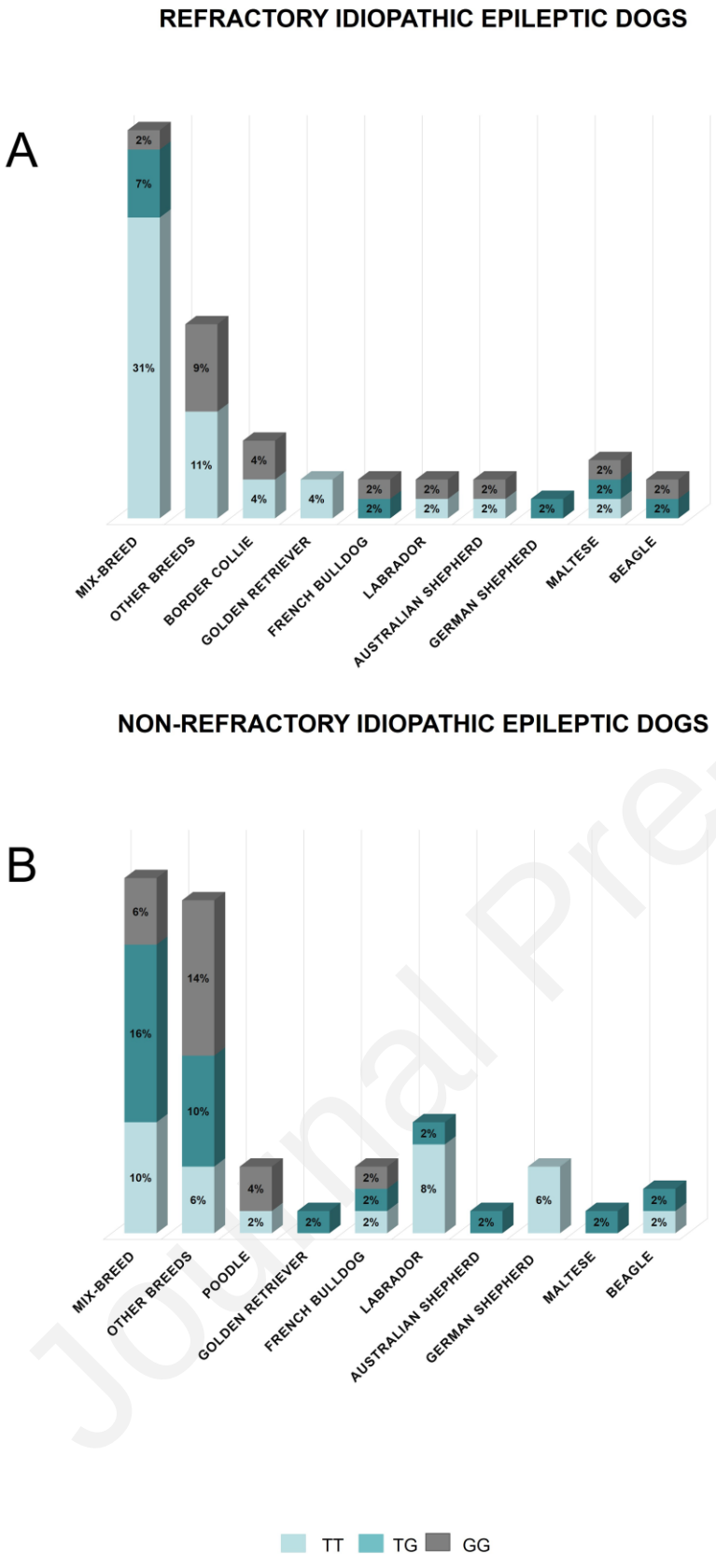

Fig. 2. Allelic frequency of *ABCB1* c.-6- 180T>G in (A) dogs with refractory idiopathic epilepsy (RIE; *n*=45) and (B) dogs without RIE (*n*=50).