Charcot-Marie-Tooth type 4B2 demyelinating neuropathy in miniature Schnauzer dogs caused by a novel splicing *SBF2 (MTMR13)* genetic variant: a new spontaneous clinical model

Nicolas Granger [Corresp., 1, 2] , **Alejandro Luján Feliu-Pascual** [3] , **Charlotte Spicer** [4] , **Sally Ricketts** [5] , **Rebekkah Hitti** [5] ,
Oliver Forman [5] , **Joshua Hersheson** [4] , **Henry Houlden** [4]

[1] Royal Veterinary College, University of London, Hatfield, United Kingdom

[2] Bristol Veterinary Specialists, CVS Referrals, Bristol, United Kingdom

[3] Neurology / Neurosurgery Service, Aúna Especialidades Veterinarias, Valencia, Spain

[4] Department of Molecular Neuroscience, UCL Institute of Neurology & National Hospital for Neurology and Neurosurgery & London, London, United Kingdom

[5] Kennel Club Genetics Centre, Animal Health Trust, Newmarket, United Kingdom

Corresponding Author: Nicolas Granger
Email address: nicolasgranger@rvc.ac.uk

Background Charcot-Marie-Tooth (CMT) disease is the most common neuromuscular disorder in humans affecting 40 out of 100,000 individuals. In 2008, we described the clinical, electrophysiological and pathological findings of a demyelinating motor and sensory neuropathy in Miniature Schnauzer dogs, with a suspected autosomal recessive mode of inheritance based on pedigree analysis. The discovery of additional cases has followed this work and led to a genome-wide association mapping approach to search for the underlying genetic cause of the disease.

Methods For genome wide association screening, genomic DNA samples from affected and unaffected dogs were genotyped using the Illumina CanineHD SNP genotyping array. *SBF2* and its variant were sequenced using primers and PCRs. RNA was extracted from muscle of an unaffected and an affected dog and RT-PCR performed. Immunohistochemistry for myelin basic protein was performed on peripheral nerve section specimens.

Results The genome-wide association study gave an indicative signal on canine chromosome 21. Although the signal was not of genome-wide significance due to the small number of cases, the *SBF2* (also known as *MTMR13)* gene within the region of shared case homozygosity was a strong positional candidate, as 22 genetic variants in the gene have been associated with demyelinating forms of Charcot-Marie-Tooth disease in humans. Sequencing of *SBF2* in cases revealed a splice donor site genetic variant, resulting in cryptic splicing and predicted early termination of the protein based on RNA sequencing results.

Conclusions This study reports the first genetic variant in Miniature Schnauzer dogs responsible for the occurrence of a demyelinating peripheral neuropathy with abnormally folded myelin. This discovery establishes a genotype / phenotype correlation in affected Miniature Schnauzers that can be used for the diagnosis of these dogs. It further supports the dog as a natural model of a human disease; in this instance Charcot-Marie-Tooth disease. It opens avenues to search the biological mechanisms responsible for the disease and to test new therapies in a non-rodent large animal model. In particular, recent gene editing methods that led to the restoration of dystrophin expression in a canine model of muscular dystrophy could be applied to other canine models such as this before translation to humans.

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2 miniature Schnauzer dogs caused by a novel splicing *SBF2*
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6 **Authors**

8 Nicolas Granger[1,2], Alejandro Luján Feliu-Pascual[3], Charlotte Spicer[4], Sally L. Ricketts[5],
9 Rebekkah J. Hitti[5], Oliver P. Forman[5], Joshua Hesheson[4], Henry Houlden[4]
10
11
12 [1]: The Royal Veterinary College, University of London, Hawkshead Lane, Hatfield, Hertfordshire,
13 United Kingdom
14
15 [2]: CVS referrals, Bristol Veterinary Specialists at Highcroft, Bristol, United Kingdom.
16
17 [3]: Neurology / Neurosurgery Service, Aúna Especialidades Veterinarias, Algepser 22-1,
18 Paterna, Valencia, Spain.
19
20 [4]: Department of Molecular Neuroscience, UCL Institute of Neurology & National Hospital for
21 Neurology and Neurosurgery & London, United Kingdom.
22
23 [5]: Kennel Club Genetics Centre, Animal Health Trust, Kentford, Newmarket, Suffolk, United
24 Kingdom.
25
26 Corresponding Author:
27
28 Dr Nicolas Granger
29
30 The Royal Veterinary College, University of London, Hawkshead Lane, Hatfield, Hertfordshire,
31 United Kingdom & CVS referrals, Bristol Veterinary Specialists at Highcroft, Bristol, United
32 Kingdom.
33 Email address: nicolasgranger@rvc.ac.uk

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40 **Abstract**

41

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43 in humans affecting 40 out of 100,000 individuals. In 2008, we described the clinical,

44 electrophysiological and pathological findings of a demyelinating motor and sensory neuropathy

45 in Miniature Schnauzer dogs, with a suspected autosomal recessive mode of inheritance based

46 on pedigree analysis. The discovery of additional cases has followed this work and led to a

47 genome-wide association mapping approach to search for the underlying genetic cause of the

48 disease.

49

50 **Methods** For genome wide association screening, genomic DNA samples from affected and

51 unaffected dogs were genotyped using the Illumina CanineHD SNP genotyping array. *SBF2* and

52 its variant were sequenced using primers and PCRs. RNA was extracted from muscle of an

53 unaffected and an affected dog and RT-PCR performed. Immunohistochemistry for myelin basic

54 protein was performed on peripheral nerve section specimens.

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56 **Results** The genome-wide association study gave an indicative signal on canine chromosome

57 21. Although the signal was not of genome-wide significance due to the small number of cases,

58 the *SBF2* (also known as *MTMR13)* gene within the region of shared case homozygosity was a

59 strong positional candidate, as 22 genetic variants in the gene have been associated with

60 demyelinating forms of Charcot-Marie-Tooth disease in humans. Sequencing of *SBF2* in cases

61 revealed a splice donor site genetic variant, resulting in cryptic splicing and predicted early

62 termination of the protein based on RNA sequencing results.

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64 **Conclusions** This study reports the first genetic variant in Miniature Schnauzer dogs

65 responsible for the occurrence of a demyelinating peripheral neuropathy with abnormally folded

66 myelin. This discovery establishes a genotype / phenotype correlation in affected Miniature

67 Schnauzers that can be used for the diagnosis of these dogs. It further supports the dog as a

68 natural model of a human disease; in this instance Charcot-Marie-Tooth disease. It opens

69 avenues to search the biological mechanisms responsible for the disease and to test new

70 therapies in a non-rodent large animal model. In particular, recent gene editing methods that led

71 to the restoration of dystrophin expression in a canine model of muscular dystrophy could be

72 applied to other canine models such as this before translation to humans.

73

Introduction

75

76   Charcot-Marie-Tooth (CMT) disease is the most common neuromuscular disorder of the human

77   population, affecting 40 out of 100,000 individuals in Europe and the United States [1-3] and

78   more than 2.6 million people worldwide [4]. It was jointly described by Drs. Charcot and Marie in

79   Paris and Dr. Tooth in Cambridge in 1886 [5]. It is now well established that CMT disease refers

80   to a wide group of degenerative motor and sensory neuropathies, 70% of which are hereditary

81   [2, 6, 7]. More than 60 genes encompassing ~900 genetic variants have been implicated in the

82   pathogenesis of CMT [8] offering the potential for further research to elucidate alterations in

83   biological cascades causing the neuropathies. However, few advances have been made so far

84   and CMT remains a slowly progressive and incurable disease. Current research is focused on

85   the use of rodent transgenic models, which have several limitations. They are difficult and time

86   consuming to obtain and the expected phenotype cannot be guaranteed [8], limitations that are

87   not present with spontaneously occurring animal models.

88

89   In companion dogs, CMT-like diseases naturally occur, as evidenced by the phenotypic

90   descriptions made by veterinarians in 22 breeds up until 2011 [, 10]. We have now entered a

91   phase where veterinary researchers and canine geneticists are taking advantage of next-

92   generation sequencing techniques in order to identify the molecular defects underlying these

93   diseases in dogs, sometimes with a very small number of affected individuals [11, 12]. This

94   promises to lead to an exponential unravelling of genetic variants in animals spontaneously

95   affected by inherited neurological diseases.

96

97   In support of this, in the last decade, several variants within five different genes have been

98   reported in eight canine breeds affected by inherited polyneuropathies similar to CMT disease in

99   humans. First found in 2009, a deletion in the mitochondrial *tRNATyr* gene in Golden Retrievers

100   causes a sensory neuropathy [13]. Following on, variants of the *NDRG1* gene, implicated in

101   myelination, cause an early-onset progressive polyneuropathy in Alaskan Malamutes [14] and a

102   hereditary motor and sensory polyneuropathy in Greyhounds [15]. The *ARHGEF10* gene,

103   involved in neuronal growth and axonal migration, is mutated in some Leonberger and Saint

104   Bernard dogs with severe juvenile-onset polyneuropathy and laryngeal paralysis [16] whereas a

105   variant of the *GJA9* gene, which is part of the connexin gap junction family proteins, causes a

106   late onset polyneuropathy with laryngeal paralysis in Leonberger dogs [17]. Finally, the

107 *RAB3GAP1* gene, involved in Rab proteins' function in membrane trafficking in the endoplasmic
108 reticulum, axonal transport, autophagy and synaptic transmission, has a single nucleotide
109 deletion in Black Russian terrier dogs [18] and a SINE insertion in Alaskan Huskies [19], leading
110 to juvenile-onset, laryngeal paralysis, polyneuropathy and ocular changes similar to Warburg
111 micro syndrome in humans. In Rottweilers, a variant in the same *RAB3GAP1* gene causes
112 neuronal vacuolation and spinocerebellar degeneration [20]. Older neuropathies, such as the
113 well-known inherited sensory and autonomic in the Border Collie first described in 1987 [21],
114 have also benefited from genetic advances. In 2016 one causal genetic variant of *FAM134B*,
115 which encodes a *cis*-Golgi protein found in sensory and autonomic ganglion neurons, has been
116 discovered [22] and two-years later, this mutation was also described in two mixed breed dogs
117 [23]. In four other breeds, the German short-haired Pointer, the English Pointer, the English
118 Springer Spaniel and the French Spaniel, some known since 1964, a similar inherited sensory
119 and autonomic exist and found to be linked to a genetic variation of the long-non-coding RNA
120 located upstream of the *Glial cell-Derived Neurotrophic Factor* gene, coding for a neurotrophic
121 factor involved in neuronal development and adult neuronal survival [24]. The discovery of this
122 genetic variant is particularly relevant to hereditary sensory and autonomic neuropathies in
123 humans where it is not known.
124
125 In 2008, we described the clinical, electrophysiological and pathological findings of a
126 demyelinating hereditary motor and sensory neuropathy in Miniature Schnauzer dogs,
127 characterized by the presence of focally folded myelin sheaths also known as *tomacula* and
128 segmental demyelination [25]. After exclusion of variants in the *PMP22* and *P0* myelin genes by
129 direct sequencing, the aim of this study was to demonstrate that the demyelinating hereditary
130 motor and sensory neuropathy in Miniature Schnauzer dogs was genetic in origin by conducting
131 a genome-wide association study (GWAS) and resequencing of candidate genes. From these
132 investigations, a splice variant in the *SBF2* (SET-binding factor 2), also known as *MTMR13*
133 (myotubularin-related protein-13) gene was identified. We will be using the official HUGO Gene
134 Nomenclature Committee gene name *SBF2* throughout the remainder of the manuscript to refer
135 to the *SBF2/MTMR13* gene.
136
137
138 **Materials & Methods**
139
140 **Study samples**

141 All dogs were examined and investigated by veterinary neurologists. Blood was collected from
142 Miniature Schnauzers pre-mortem and tissue samples were collected post-mortem after dogs had
143 been euthanized on welfare grounds. Euthanasia was carried out solely to alleviate suffering and
144 no healthy individuals were sacrificed for use in this study. Euthanasia was carried out in
145 accordance with the Veterinary Surgeons Act 1966 and under the auspices of the RCVS. All
146 samples used in this study were collected after permission had been granted by dog owners (24-
147 2018E).
148
149 **Sample DNA and RNA extraction**
150 All DNA samples were collected from privately owned pet dogs by blood or tissue extraction using
151 the QIAamp midi or mini DNA extraction kit (Qiagen). Affected dogs are described below and some
152 have been previously reported. Controls were Miniature Schnauzer dogs that were reported as
153 clinically healthy with no sign of neuropathy based on our neurological examination of these cases
154 (n=7), or apparently healthy Miniature Schnauzers with no owner report of neurological disorder
155 (n=224). Cerebellum or muscle tissue samples were collected post-mortem and DNA and total
156 RNA extracted using the RNeasy midi kit (Qiagen). Genomic DNA from AHT control dogs utilized
157 for this study were derived from buccal swab samples or from residual blood drawn for diagnostic
158 veterinary purposes as part of the dog's veterinary care. Samples were taken following informed
159 and written owner consent. Sample collection for genetic research has been approved by the
160 Animal Health Trust Ethics Committee (24-2018E).
161
162 Total RNA was extracted from homogenized muscle from an affected dog with two copies of the
163 *SBF2* variant and a control dog using a miRNeasy Mini Kit (Qiagen, Chatsworth, CA) according to
164 the manufacturer's instructions. First-strand cDNA synthesis was then carried out using SuperScript
165 II Reverse Transcriptase First-Strand Synthesis System for RT-PCR (Invitrogen / Life
166 Technologies). The synthesizing conditions included initial 5 minutes' incubation at 65°C with dNTP
167 and a mix of random hexamers/oligo dT primers, after which tubes were placed on ice for at least 1
168 minute. cDNA synthesis mix (5 x First-Strand Buffer, 0.1M DTT, RNase OUT) was added and
169 incubated at 25°C for 2 minutes. SuperScript II was added and tubes incubated at 25°C for 10
170 minutes, followed by 42°C for 50 minutes. The reaction was terminated by incubation at 70°C for 5
171 minutes.
172
173 **GWAS analysis**

174   Genomic DNA samples from the first two Miniature Schnauzers diagnosed with a peripheral

175   neuropathy in France by one author (NG), and 39 Miniature Schnauzer controls were genotyped

176   using the Illumina CanineHD SNP genotyping array that comprises 173,662 SNPs (*i.e.* a total of

177   41 dogs). The SNP genotyping dataset was analyzed for association using the statistical

178   package PLINK [26]. As part of the 41 dogs, a set of nine dogs (comprising the two affected

179   cases and seven controls) and a set of 32 controls were genotyped by two centers; University

180   College London (JH, HH) and Animal Health Trust (SLR, RJH, OPF) respectively. To preserve

181   data quality SNP QC was conducted in each dataset independently before merging. Sample call

182   rates for all individuals were >99%. SNP quality control filtering excluded SNPs with a minor

183   allele frequency of less than 5% and a genotyping call rate of less than 97% in each

184   independent dataset. This resulted in 74,374 SNPs for association analysis in the merged

185   dataset. Multidimensional scaling analysis did not indicate the presence of population

186   stratification between the two sets (Supplementary file 1). Later, a further 192 Miniature

187   Schnauzers (Animal Health Trust) with no report of the disorder were included as a follow-up set

188   along with six other affected cases (five from Spain, one from Belgium), altogether leading to a

189   set of eight affected dogs and 231 controls.

190   Due to the very small sample size and disproportionate case-control ratio we conducted the

191   GWAS analysis using a Fisher's exact test with 100,000 permutations (max(T) permutation

192   procedure) to account for multiple testing. (The threshold for genome-wide statistical association

193   after permutation is 1.3.)

194

195   ***SBF2* resequencing**

196   This was done using the two originally described French cases; five affected cases available from

197   Spain; and one case from Belgium – these dogs presented with similar neurological and

198   electrophysiological signs as originally described. Genomic DNA was amplified using a standard

199   touchdown PCR reaction. Cycling conditions were as follows: 94°C (5 minutes); 94°C (30 seconds),

200   60°C (30 seconds), 72°C (45 seconds) for 25 cycles; 94°C (30 seconds) 50°C (30 seconds) 72°C

201   (45 seconds) for 13/18 cycles, 72°C (10 minutes). Primers for each of 35 coding exons with flanking

202   intronic regions were designed with PrimerZ (http://genepipe.ngc.sinica.edu.tw/primerz/) (see

203   sequences in Supplementary file 2). Enzymatic clean-up with Exonuclease I and

204   *FastAP* Thermosensitive Alkaline Phosphatase (Thermo Scientific) was carried out and subsequent

205   sequencing performed. PCR primers were used for sequencing along with BigDye Terminator v.1.1

206   cycle sequencing kit (Applied Biosystems). Cycling conditions were as follows: 94°C (1 minute);

207   94°C (30 seconds), 50°C (15 seconds), 60°C (4 minutes) for 25 cycles. Sequencing reaction

208   products were purified using sephadex columns and then run on a 3730xl DNA Analyzer. The

209   resulting data sequences were aligned to the canine *SBF2* reference sequence (CanFam 3.1)

210   retrieved from Ensembl (ENSCAFG00000007500; ensembl.org) and manually analyzed using

211   Sequencher v. 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA). Gene Runner was used to

212   investigate the amino acid sequence.

213

214   **Variant genotyping and statistical analysis**

215   The *SBF2* variant was genotyped using an allelic discrimination assay using an ABI StepOne real-

216   time thermal cycler. Primers and allelic discrimination probes were designed using Primer3 [27] and

217   obtained from Integrated DNA Technologies (IDT). Primer sequences were:

218   TGGGAGAGTGGAAGCAACAG (forward); GGAGTGTCTCTGTATGTGCACATT (reverse); 5'-

219   FAM- TTGTCACAAACAGTTACCAA-NFQ-3' (mutant); 5'-VIC- TTGTCACAAACAGGTACCAA-

220   NFQ-3' (wildtype). Individual probe assays were re-suspended in ultrapure water to a 40X mix and

221   combined. Allelic discrimination reactions were prepared using KAPA probe fast (KAPA

222   Biosystems) with the 40X probe mix. Thermal cycling conditions were: 25°C for 30 seconds; 95°C

223   for 3 minutes; followed by 40 cycles of 95°C for 3 seconds; and 60°C for 10 seconds, then the

224   reaction held at 25°C for 30 seconds.

225

226   As the variant was rare in our sample set, a subset of genotypes was verified by Sanger

227   sequencing. Primer sequences were AACCTCATGGTCAACCTGCT (forward) and

228   TGCACAGCAGTATTTGCCTAA (reverse). PCR cycling consisted of an initial 95°C denaturation

229   step for 5 minutes, followed by 35 cycles of 95°C for 30 seconds; 60°C for 30 seconds; and 72°C

230   for 1 minute. Final elongation was 72°C for 5 minutes. PCR products were purified using 96-well

231   multiscreen PCR plates (Merk Millipore) and sequenced in both directions using BigDye Terminator

232   v3.1 chemistry (Applied Biosystems) and an ABI 3130XL Genetic Analyzer (Applied Biosystems).

233   Sequences were aligned and manually called using the Staden package [28].

234

235   The association between demyelinating neuropathy and genotype was assessed using the Fisher's

236   exact test.

237

238   **Immunohistochemistry of peripheral nerve sections**

239   Peripheral nerve specimens originating from surgical biopsies at the time of diagnosis were

240   embedded in OCT and snap frozen in isopentane pre-cooled with liquid nitrogen and stored at -

241   80°C. Cryostat sections of 30 μm were then cut and mounted on histopathology slides and air

242   dried for 30 minutes at 37°C. The sections were stained for myelin basic protein as follows:

243   incubation in a blocking solution of 10% normal goat serum 0.1M PBS and 0.3% t-

244   octylphenoxypolyethoxyethanol for an hour at room temperature; incubation with primary

245   antibody (anti-myelin basic protein monoclonal antibody, 1:100, Chemicon) diluted in 0.1M PBS

246   containing normal goat serum overnight at 4°C; washing 3 times with 0.1 M PBS for 5 minutes;

247   incubation with a secondary antibody (Alex Fluor 488, goat anti-rat, ThermoFischer Scientific)

248   for 2 hours, in the dark, at room temperature. The sections were then examined under a

249   fluorescent microscope.

250

251

252   **Results**

253

254   **Disease phenotype**

255   The phenotype of this inherited demyelinating peripheral nerve disease was previously reported

256   and affected dogs share familial links, based on the pedigree analysis of 32 dogs (see Figure 2

257   from Vanhaesebrouck et al.) [25]. Briefly, at a young age (< 2 years), affected Miniature

258   Schnauzers presented regurgitations caused by mega-esophagus and inspiratory dyspnea caused

259   by laryngeal paralysis. Electrophysiological studies revealed marked slowing of motor and sensory

260   nerve conduction velocities (~20m/s), although some nerves had preserved conduction velocities.

261   Although the prognosis remains guarded, long survival is observed with dogs alive > 3 years

262   following diagnosis. Teased nerve fibers presented variable thickness of the myelin sheath with

263   characteristic '*tomacula*' and area of segmental demyelination. Toluidine-blue-stained semi-thick

264   resin-embedded sections demonstrated loss of myelinated fibers, thinly myelinated fibers and

265   redundant myelin loops again characteristic of the presence of *tomacula*, as previously reported by

266   Vanhaesebrouck et al. [25]. Muscle biopsy specimens appeared normal when sampled [25].

267   Immunohistochemistry of peripheral nerve sections stained for myelin basic protein (Fig. 1)

268   demonstrate the loss of normal myelin architecture in affected cases.

269

270   **Mapping of the causative variant**

271   The genome-wide association study was conducted using two cases originally described in

272   2008 [25] and 38 apparently healthy breed-matched controls (one control sample did not work

273   during testing). The strongest associated SNP was located on canine chromosome 21

274   (BICF2S2377579 – CanFam 3.1 position chr21: 34,045,084) (Fig. 2), although this did not reach

275   genome-wide statistical association due to the small dataset and concurrent lack of statistical

276   power. Interrogation of the SNP genotype distributions across the associated region identified a

277 run of shared homozygosity in cases spanning from chr21:18,552,117-46,804,230

278 (CanFam3.1). The most obvious candidate gene in this region was the *SBF2* gene, which

279 causes CMT in human patients with a clear phenotype.

280

281 Resequencing of the *SBF2* gene was initially performed using one of the two affected case and

282 one control. This revealed a homozygous +1 splice genetic variant in exon 19 (c.2363+1 G>T;

283 chr21:33,080,022 C>A CanFam3.1) in the affected case in comparison to the control (Fig. 3).

284 No further variants were found in the entire coding sequence. This putative disease-causing

285 c.2363+1 G>T variant was assessed in the initial GWAS set (2 affected and 39 controls) *plus* six

286 additional affected cases and a further 192 Miniature Schnauzers with no report of the disorder,

287 demonstrating a highly significant statistical association (see Table 1) in comparison to the top

288 GWAS SNP. This identified 10 heterozygotes and 182 dogs with a homozygous wildtype

289 genotype in the added 192 controls and 38 homozygous wildtype genotypes in the GWAS set

290 (one control from this set did not work); but no dogs homozygous for the variant in the control

291 set. The 6 affected cases, 5 from Spain and one from Belgium, were mutant homozygous dogs.

292 Screening for the variant in a dataset of 802 whole genome sequences from 162 purebred

293 dogs/mixed breed dogs/wolves identified two Miniature Schnauzers that were heterozygous for

294 the variant and one mutant homozygote; the remaining 799 dogs were clear (Supplementary file

295 3). On follow-up investigation the mutant homozygous dog was affected with neonatal lethal

296 spondylocostal dysostosis, hence no clinical data relevant to the demyelinating neuropathy

297 phenotype was available [29]. The original two GWAS cases were found in France and the

298 mutant homozygote identified through genome data was of Australian origin, although the sire

299 was from Sweden. The additional six affected cases were from Spain and Belgium. This

300 tentatively suggests presence of the variant in different regions of the globe.

301

302 **Effect of *SBF2* variant on mRNA splicing and coding sequence**

303 To analyze the consequence of this splicing acceptor site variant on *SBF2* pre-mRNA splicing,

304 RNA was extracted from dissected muscle of an unaffected and an affected dog (the case found

305 in Belgium) and RT-PCR performed. The resulting transcripts were sequenced which revealed

306 the presence of a 40-bp deletion at the 3' site of exon 19 due to the induction of a cryptic splice

307 site in this exon (Fig. 4A). The *SBF2* genetic variant and resulting 40-bp deletion leads to a

308 premature stop codon (p.G775V*fs**5) and truncates the protein by 1070 amino acids (Fig. 4B).

309

310

Discussion

311 **Discussion**

312 We previously described a suspected inherited demyelinating peripheral neuropathy in

313 Miniature Schnauzer dogs and highlighted its similarities with some demyelinating forms of CMT

314 in humans [25, 30]. Mainly, we discovered in the dog the presence of specific neuropathological

315 features of the peripheral nerves, such as abnormal folding of peripheral myelin around the

316 axons termed *tomacula*, as originally described in humans with certain demyelinating

317 neuropathies [31, 32]. An inherited trait for the canine disease equivalent was suggested based

318 on pedigree analysis of affected dogs. Our findings led us to suspect that variants of genes

319 coding for: (i) myelin proteins (such as *PMP22*, *P0*, *periaxin*); (ii) intracellular Schwann cell

320 proteins involved in the synthesis, transport and degradation of myelin proteins including the

321 myotubularin-related (MTMR) proteins; and (iii) regulating myelin gene transcription proteins

322 (such as *EGR2*), might be responsible for the disease [33, 34]. In particular, variants of genes

323 coding for the MTMR proteins and leading to abnormal myelin compaction have been described

324 in CMT type 1, type 4B1 (variants in *MTMR2*) and type 4B2 (variant in *SBF2 (MTMR13)*,

325 *MTMR2* regulatory binding partner) [33, 35-51]. In this study we have mapped demyelinating

326 neuropathy in the Miniature Schnauzer to canine chromosome 21 and have subsequently

327 identified a genetic variant in and exon 19 of *SBF2* that shows strong segregation with the

328 disease in this breed. Variants of the *SBF2* gene are known in humans to cause CMT type 4B2

329 (although a variant in exon 19 has not been found in humans). So far, thirty-seven humans with

330 twenty-six pathogenic *SBF2* variants leading to CMT type 4B2 have been reported [52], a

331 number that has dramatically increased recently with the discovery of nine new *SBF2* variants in

332 one study [49] and three new *SBF2* variants in another [48].

333 Myotubularins (MTM) form a family of 14 proteins [53] that act as phosphoinositide phosphatase

334 (PP) proteins and play a central role in neuromuscular homeostasis [39]. Some are catalytically

335 active (such as MTRM2) and dephosphorylate sub pools of phosphatidylinositols (PtdIns) [53-

336 57], while others are catalytically inactive and function as a scaffold to bind other myotubularins

337 and proteins (such as MTMR13 binds MTMR2) [58, 59]. They are implicated in a number of

338 physiological processes including cell proliferation, growth, survival, motility, cytoskeletal

339 regulation and intracellular vesicle trafficking and signaling (endosomal-lysosomal pathways)

340 [53, 60]. Their importance is highlighted by the large number of diseases associated with variant

341 of myotubularin genes [61-63], but it remains poorly understood how these variants lead to

342 disease.

343 Their ubiquitous expression, lack of functional redundancy and involvement in varied

344 pathologies suggests that their expression and regulation finely varies between organs [64, 65].

345   For example, in humans, a variant within the dDENN domain of *SBF2* leads to a demyelinating

346   neuropathy [39] and a variant within the DENN domain causes a thrombocytopenia without

347   neurological manifestations [47]. It is believed in this latter case that the genetic variant affects

348   the solubility of the SBF2 protein but that the protein conformation is preserved [47].

349   In cases with involvement of the peripheral nerves, the analysis of the type of genetic variants

350   associated with *SBF2* also provides a very good example of how variants in one gene, and

351   within a particular domain, leads to different phenotypes, in particular with additional

352   involvement of the eye. The first *SBF2* variant in humans was reported in a Turkish family and

353   involved a deletion of 1,301 bp across exons 11 and 12, removing the unique dDENN motif [39]

354   but allowing the synthesis of a partially functional *SBF2* protein highlighting the possible role of

355   dDENN in myelin compaction. This only caused a demyelinating neuropathy. Later, in a

356   Tunisian and a Moroccan family, two nonsense variants in *SBF2* were identified in exons 23

357   (2875C>T (Gln956Stop)) and 27 (3586C>T (Arg1196Stop)) [40]. These patients presented

358   juvenile onset glaucoma associated with the demyelinating neuropathy, a phenotype that was

359   also found in a Japanese family affected by another nonsense variant (1459C>T (Arg487Stop))

360   in exon 14 of *SBF2* gene [41]. These variants resulted in a truncated protein with deletion of the

361   GRAM, myotube-related and PH domains and complete loss of function. Finally, with variants of

362   the inactive phosphatase domain of *SBF2*, leading to presence of an abnormal but likely

363   partially functional *SBF2* protein, human patients have a demyelinating peripheral neuropathy

364   with or without glaucoma [42, 49]. The occurrence of ocular anomalies in association with

365   peripheral neuropathy is also present in the recent report of an inherited peripheral neuropathy

366   in Black Russian terrier dogs [18].

367

368   The finding of a variant in *SBF2* in the dogs of this report strikingly exemplifies the wide role of

369   myotubularin proteins since a variant of *MTM1* has also been reported in a group of Labrador

370   Retrievers, but leads to a centro-nuclear myopathy [66], as it does in humans [64]. These dogs

371   are clinically normal at birth but develop generalized muscular weakness and muscle atrophy at

372   ~7 weeks of age and die in their first six months of life. *MTM1* is specifically involved in

373   endosomal and membrane trafficking pathways, and late maturation and maintenance of T-

374   tubules. In affected dogs, the structure of the T tubule network is affected, leading to

375   disorganization of the muscle triads and their maintenance.

376

377   This new Miniature Schnauzer canine model allows useful comparisons between the human

378   and canine disease for several reasons. First, this is a spontaneous (*i.e.* naturally occurring)

379 model of a demyelinating neuropathy with focally folded myelin. This is important because the

380 phenotype is likely more relevant and closer to the disease observed in humans, knowing the

381 limitations of the rodent models. *SBF2* deficient mice have been created by inserting a gene-

382 trap vector following exon 14 of *SBF2*, leading to lack of expression of the PH-GRAM and

383 phosphatase domains and coiled-coil motif of *SBF2* [67], which are the domains involved in

384 binding, catalyzing and mediating interactions between active and inactive myotubularins [68,

385 69]. Although *SBF2* is broadly expressed in the mouse [70], the phenotype of this model differs

386 from the naturally occurring disease since no reduction in the sciatic nerve conduction velocity

387 was observed at 12 months of age in mice (this is also the case in a mouse model of the

388 MTMR2 neuropathy [71]), which markedly contrasts with the profound slowing of nerve

389 conduction velocity observed in dogs [35] and humans [39-42]. Clinically, these mice are not

390 distinguishable from wild type animals but behavior analysis with the rotarod test identified

391 deficient mice [67].

392 Another group of researchers created a similar mouse mutant by inserting a gene-trap plasmid

393 following exon 17 [72], resulting in a similar defect as described above. Again, these animals did

394 not show obvious behavioral abnormalities, but in contrast to the model from Tersar et al. [67],

395 showed reduced motor nerve conduction velocities (in the range of 20m/s). These two examples

396 illustrate the complexity of establishing engineered animal models in the laboratory because the

397 clinical phenotype obtained is not predictable, or is variable or lacking. In contrast, affected

398 Miniature Schnauzer dogs present to veterinary hospitals with clear clinical signs, marked

399 electrophysiological abnormalities and characteristic pathological features. As shown above, the

400 variant described can be directly compared to variants affecting some human families, where

401 protein abnormalities are more likely to be similar. Altogether, the signs observed in dogs

402 provide means to follow-up the disease progression and to trial novel therapies. Secondly, the

403 dog model could be used for *in vitro* studies, for example using fibroblast cultures that can be

404 easily obtained from skin biopsies. In one study [73] using the Robinson's mouse model [72],

405 the consequences of *SBF2* variant on the endo-lysosomal pathway has been investigated and it

406 was found that loss of *SBF2* does not dysregulate the endo-lysosomal membrane system in

407 Schwann cells. Therefore, there is a need to further identify the impact of *SBF2* loss on the

408 trafficking of specific cargo proteins. The prevalence of the disease in Miniature Schnauzers is

409 currently not known but there is likely a pool of animals that could be available for tissue

410 sampling and further *in vitro* research. Future studies could focus on the identification of specific

411 membrane proteins that may be abnormally trafficked in the absence of *SBF2*, as well as on

412 defining how phosphatidylinositols are dysregulated by the loss of these proteins [74]. Finally, it

413 should be mentioned that, although spontaneous canine models are appealing, they come with

414 limitations. In particular, their remain unknown as to how many cases could be accessed for

415 research. The use of companion dogs in research requires strict ethical safeguards, and

416 imposes limitation to access to tissue (including post-mortem) and interventions that can be

417 performed. The cost of developing colonies of affected dogs within veterinary laboratories and

418 cost of running clinical trial is also greater than the cost of working with rodents.

419

420

421 **Conclusions**

422

423 In conclusion, we have identified a splicing variant in *SBF2* as the most likely cause for the

424 demyelinating peripheral neuropathy observed in Miniature Schnauzer dogs. This provides the

425 first naturally occurring large animal model of a demyelinating form of CMT disease, 4B2.

426 The discovery and description of naturally occurring models of CMT diseases open great

427 research avenues. They render possible *in vitro* characterization of molecular pathways and

428 functional work, such as with the use of fibroblasts obtained from the skin or nerve

429 transcriptome analysis. The large pool of affected dogs and expansion of veterinary hospitals

430 could allow collection of samples for this research and could pave the way for testing new

431 therapies in canine models, as exemplified by the recent use of gene editing to restore

432 dystrophin expression in a canine model of muscular dystrophy [75]. This strategy could be

433 applied to many genetic diseases, such as the demyelinating peripheral neuropathy presented

434 here, while the use of a larger animal model allows real-life quantification of the benefit of

435 proposed therapies.

436

437

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439

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455

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707

Figure 1

Immunofluorescent staining for myelin basicprotein in canine peripheral nerves.

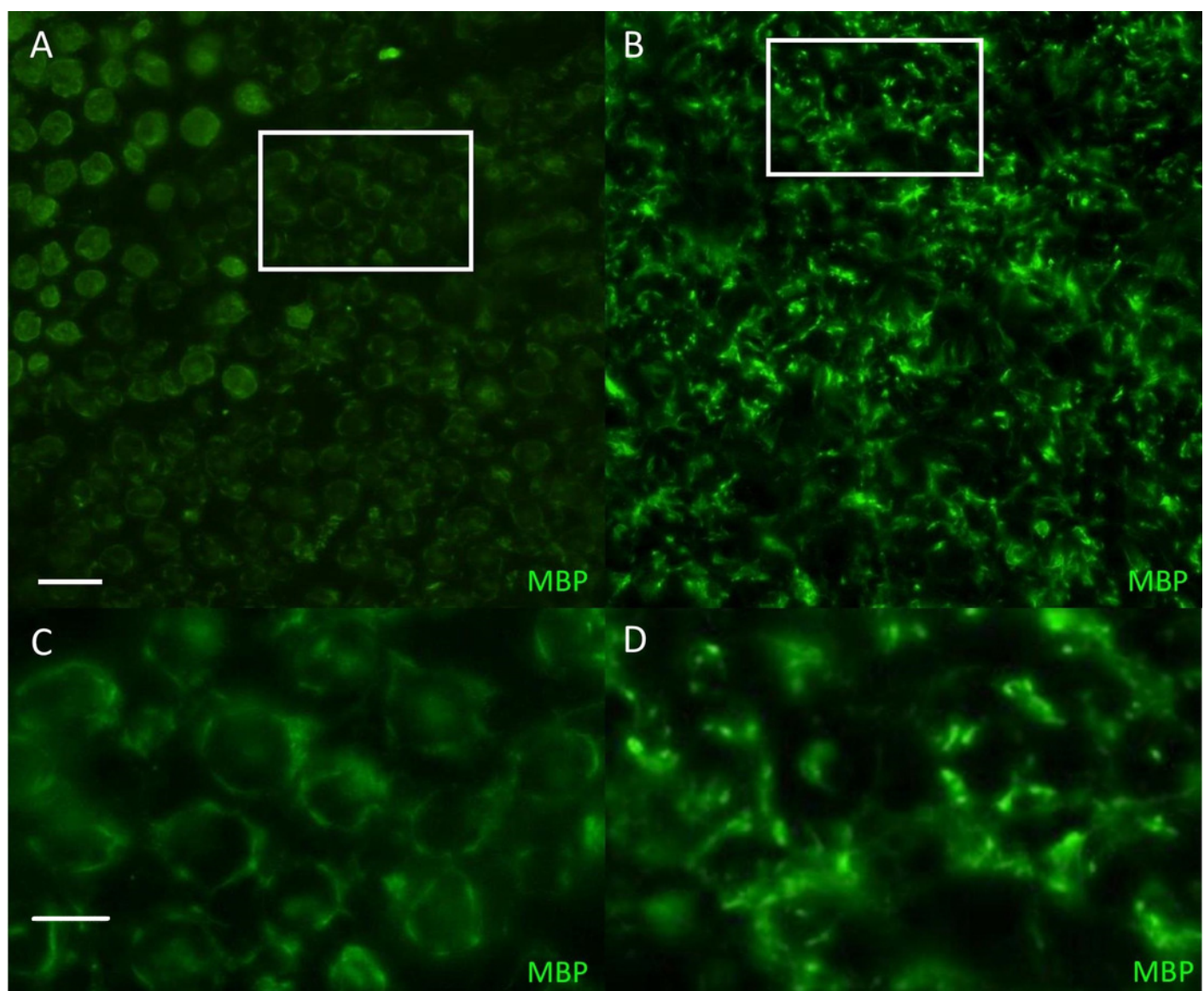
(A, C) Transverse sections (30 µm) obtained from the tibial nerve of a normal Miniature Schnauzer dog stained for myelin basic protein (green) - note the preservation of the peripheral myelin architecture delineating axons within the fascicle; (B, D) Transverse sections (30 µm) obtained from the tibial nerve of an affected Miniature Schnauzer dog - the myelin has lost its organization. C and D are magnified images of A and B white rectangles respectively. Scale bar A, C = 20 µm; scale bar B, D = 10 µm.

Miniature Schnauzers genotyped for the candidate variant originating from the initial GWAS set (2 affected cases and 38 controls*), 6 other affected cases and 192 controls.

Table 1 Miniature Schnauzers genotyped for the candidate variant originating from the initial GWAS set (2 affected cases and 38 controls*), 6 other affected cases and 192 controls.

c.2363+1 genotype [a]	Case (n)	Control (n)	Total	
G/G	0	220	220	
G/T	0	10	10	
T/T	8	0	8	P-value 4.4 x 10^{-15}

[a] **T is mutant allele**

***** **NB Data on c.2363+1 genotype is missing for one control dog**

Figure 2

Manhattan plot toillustrate results of GWAS analysis

Manhattan plot on two cases and 39 controls; 74,374 SNPs. The X-chromosome is omitted. Analyses were conducted using Fisher's exact test and 100,000 permutations to correct for multiple testing using the max(T) permutation procedure in PLINK. EMP2 = corrected empirical P-value.

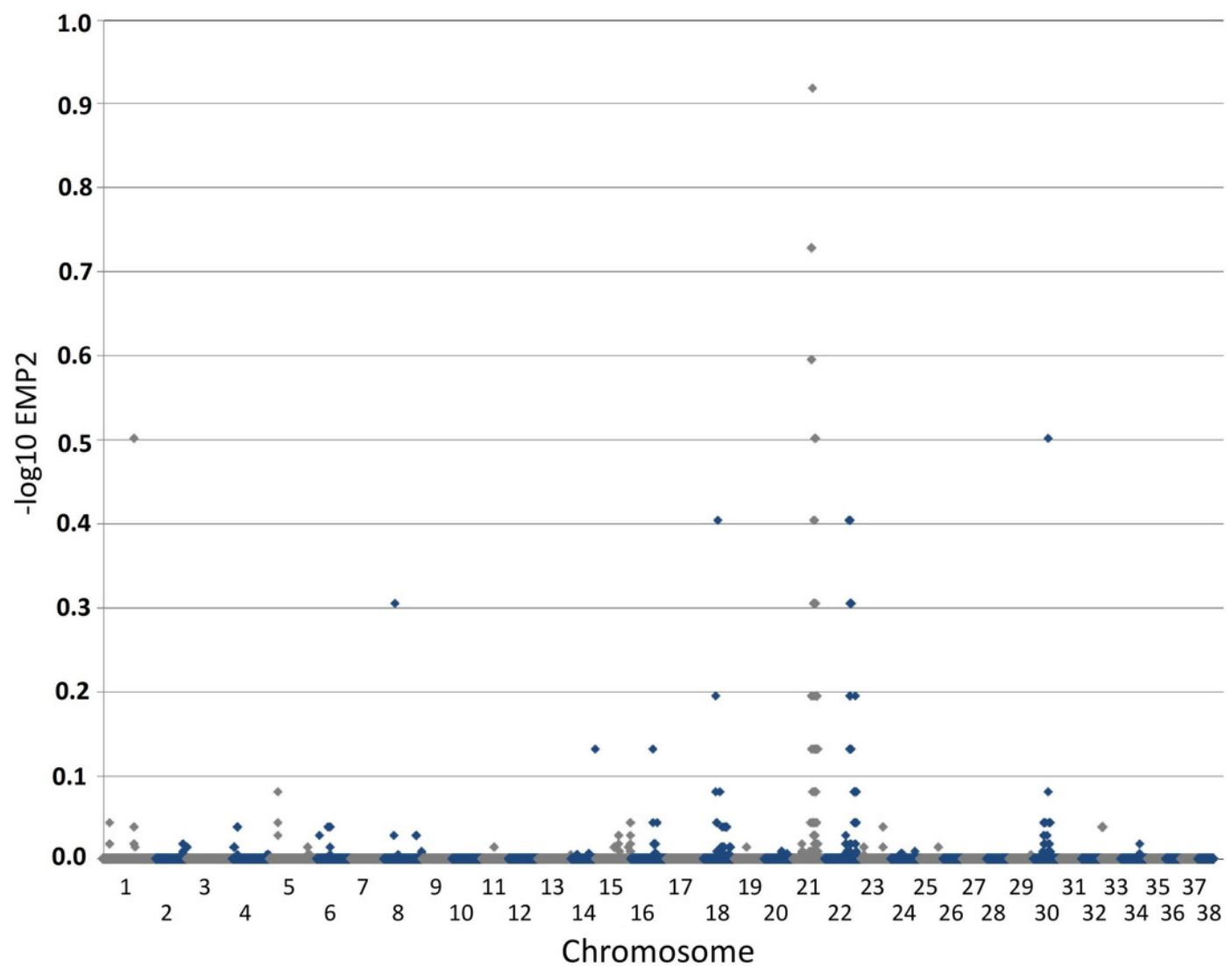



Figure 3

Sanger sequencingelectropherogram traces of the c.2363+1 G>T variant (boxed) in
*SBF2*exon 19.

Trace shows the 3' end of exon 19 and the adjacent intron from PCR products amplified from
genomic DNA.

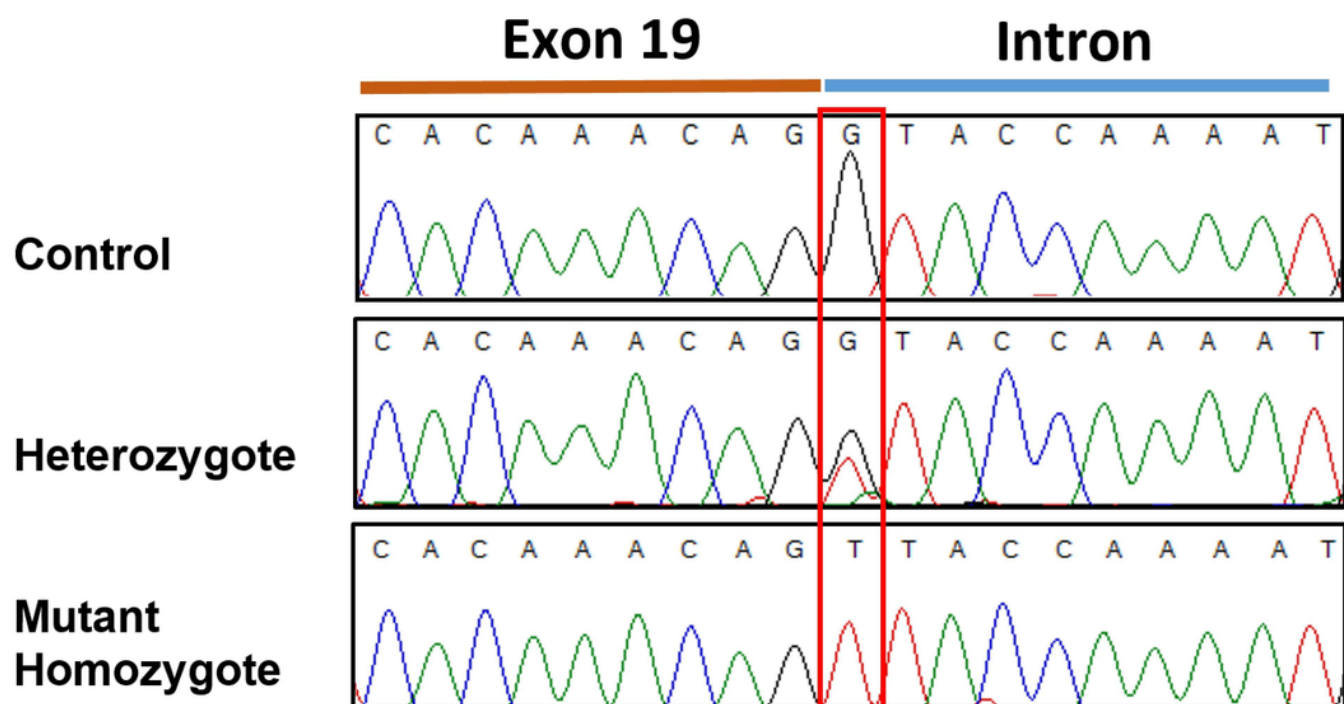



Figure 4

Electropherogram of PCR-amplified cDNA of control and affected dogs.

(A) Electropherogram of PCR-amplified cDNA from a control dog; **(B)** Electropherogram of PCR-amplified cDNA from an affected dog showing use of a cryptic splice site after disruption of the exon 20 donor site by the c.2363+1 G>T variant. The result is a 40-bp truncation of the transcript. **(C)** Normal amino acid sequence; **(D)** The cryptic splice site is positioned at c.2324-c2325 and is predicted to lead to a sequence of 5 aberrant amino acids and premature termination (p.G775V*fs*5).