

1 **A *TTPA* deletion is associated with Retinopathy with Vitamin E Deficiency (RVED) in the English**  
2 **Cocker Spaniel Dog**

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10

11 **Abstract**

12 Retinopathy with Vitamin E Deficiency (RVED) is a familial disease in the English Cocker Spaniel (ECS)  
13 dog breed. Ophthalmic abnormalities observed in RVED-affected ECS include lipofuscin granule  
14 deposition within the tapetal fundus and subsequent retinal degeneration resulting in visual deficits.  
15 Affected dogs may also exhibit neurological signs that include ataxia and hindlimb proprioceptive  
16 deficit. In all cases, circulating plasma concentrations of  $\alpha$ -tocopherol are low. This study sought to  
17 investigate the genetic basis of RVED in the ECS breed. We undertook a genome-wide association  
18 study comprising 30 ECS with normal fundic examinations aged 6 years or older (controls) and 20  
19 diagnosed with RVED (cases) and identified a statistically associated signal on chromosome 29  
20 ( $P_{\text{raw}} = 1.909 \times 10^{-17}$ ). Whole genome sequencing (WGS) of two cases identified a 102bp deletion in exon  
21 1 of the Alpha Tocopherol Transfer Protein gene (*TTPA*), truncating the protein by 34 amino acids. The  
22 c.23\_124del variant segregated with RVED in a total of 30 cases and 43 controls. Variants in *TTPA* are  
23 causal for Ataxia with Vitamin E Deficiency (AVED) in humans which is a phenotypically similar disease  
24 to RVED. The identification of the canine variant is extremely significant as the availability of a DNA  
25 test will allow for identification of presymptomatic dogs and early therapeutic intervention which may

26 prevent development of retinopathy and improve neurological signs. Breeders can also use the DNA  
27 test to efficiently eradicate the disease from this breed.

28

## 29 **Article Summary**

30 Retinopathy with vitamin E deficiency (RVED) is an inherited disease in the English Cocker Spaniel that  
31 causes blindness and ataxia. It has many similarities to ataxia with vitamin E deficiency (AVED) in  
32 humans. This study investigated the genetic basis of RVED and reveals it to be associated with a  
33 mutation in *TTPA* – the same gene that causes AVED. A DNA test has now been developed which will  
34 enable eradication of the disease from this dog breed and also allow for the identification of  
35 presymptomatic individuals. The latter would allow for early therapeutic intervention and prevention  
36 of retinal (and neurological) disease.

37

## 38 **Introduction**

39 Vitamin E comprises eight naturally occurring fat-soluble nutrients called tocopherols and  
40 tocotrienols. Vitamin E is an antioxidant that maintains cell membrane stability by prevention of lipid  
41 peroxidation (Drevon 1991; Herrera and Barbas 2001). Deficiency in vitamin E may result in pathologic  
42 changes in muscle, the reproductive tract, the central nervous system and the retina (Hayes et al.  
43 1970; McLellan et al. 2003; Stocker 2007; Traber and Head 2021). In dogs, dietary vitamin E deficiency  
44 leads to a multifocal pigmentary retinopathy (Davidson et al. 1998; Riis et al. 1981). Histologically,  
45 lipofuscin accumulation occurs within the retina and also within smooth muscle cells of the intestinal  
46 tract and within neurons of the CNS (Davidson et al. 1998; Riis et al. 1981).

47 A retinopathy with identical ophthalmoscopic signs to canine dietary vitamin E deficiency has  
48 been reported in several dog breeds including the Labrador Retriever, Golden Retriever, Briard, Border  
49 Collie, Polish Lowland Sheepdog and English Cocker Spaniel (ECS) suggesting an inherited component  
50 in these breeds (Aguirre and Laties 1976; Barnett 1969; Bedford 2009; McLellan et al. 2002; Parry  
51 1954). This retinopathy has been variably termed central progressive retinal atrophy, vitamin E

52 deficiency retinopathy and retinal pigment epithelial dystrophy (Aguirre and Laties 1976; Bedford  
53 2009; Lightfoot et al. 1996; Parry 1954; Riis et al. 1981). Histologically there is initial accumulation of  
54 lipofuscin within the retinal pigment epithelium followed by degeneration of the neurosensory retina  
55 characterised by a gradual loss of the outer nuclear layer and the subsequent atrophy and  
56 degeneration of the inner retina (Lightfoot et al. 1996; McLellan et al. 2003). Lipofuscin accumulation  
57 also occurs within smooth muscle cells throughout the body and also throughout the CNS (McLellan  
58 et al. 2003).

59         This retinopathy has been shown to be a familial disease in the ECS (McLellan et al. 2002). The  
60 age of onset is unknown as dogs tend to be presented for specialist examination quite late in the  
61 disease process, however, the mean (sd) age of affected ECS was 5.93 (2.19) years in one study  
62 (McLellan et al. 2002). Ophthalmoscopically, the disease is characterised by development of multifocal  
63 light brown pigment spots within the tapetal fundus (McLellan et al. 2002). Subsequently, these  
64 lesions coalesce to form patches and there is degeneration of the neurosensory retina manifested as  
65 retinal vascular attenuation and tapetal hyperreflectivity (McLellan et al. 2002). Affected dogs have  
66 low circulating plasma concentrations of  $\alpha$ -tocopherol ( $\alpha$ -Toc), the most abundant and biologically  
67 active form of vitamin E, in the absence of dietary deficiency or intestinal malabsorptive disease. In  
68 addition, a subsequent study of retinopathy with vitamin E deficiency, reported that several affected  
69 ECS also had clinical signs of neurological dysfunction which included ataxia, proprioceptive deficits,  
70 abnormal spinal reflexes and muscle weakness (McLellan et al. 2003).

71         In humans, inherited diseases resulting in vitamin E deficiency are most commonly associated  
72 with ataxia and occur as autosomal recessive disorders. To date, two forms of autosomal recessive  
73 ataxia due to vitamin E deficiency have been described. The first form to be described was  
74 abetalipoproteinaemia in which there is a failure of chylomicron formation and absence of  
75 lipoproteins (Burnett et al. 1993). This leads to impaired gastrointestinal absorption and severely  
76 reduced plasma  $\alpha$ -Toc concentrations. Abetalipoproteinaemia is caused by mutations in *MTTP* – the  
77 gene encoding the microsomal triglyceride transfer protein (Chardon et al. 2009; Sharp et al. 1993).

78 In the second form, ataxia with vitamin E deficiency (AVED), gastrointestinal absorption of lipids is  
79 normal but there is impaired incorporation of  $\alpha$ -Toc into lipoproteins secreted by the liver (Traber et  
80 al. 1990). AVED is caused by mutations in *TTPA* – the gene encoding  $\alpha$ -tocopherol transfer protein  
81 (Cavalier et al. 1998). Retinitis pigmentosa and pigmentary retinopathies have been associated with  
82 both forms making both *MTTP* and *TTPA* plausible candidate genes for retinopathy with vitamin E  
83 deficiency (RVED) (Abramowicz et al. 2024; Benomar et al. 2002; Ferreira et al. 2014; Iwasa et al. 2014;  
84 Mariotti et al. 2004; Matsuo et al. 1994; Nagappa et al. 2014; Shimohata et al. 1998; Yokota et al.  
85 1996; Yokota et al. 1997; Yokota et al. 2000).

86 In this study we investigated the molecular basis of RVED in the ECS using a combination of  
87 genome-wide association and whole genome sequencing strategies. Our ultimate aim was to develop  
88 a molecular test for breeders to use as a tool to eradicate the disease from the breed. A DNA test  
89 would also allow for the identification of young, presymptomatic individuals for early therapeutic  
90 intervention with potential avoidance of retinal and neurological disease.

91

## 92 **Materials and Methods**

### 93 **Sample collection**

94 Dog owners and veterinary ophthalmologists submitted ECS DNA samples to the Canine Genetics  
95 Centre (previously based at the Animal Health Trust, Newmarket, UK) as buccal mucosal swabs or  
96 residual blood samples, with owner consent (Ethical approval by Animal Health Trust Clinical Research  
97 Ethics Committee Project No 24-2018E (2018) and University of Cambridge Department of Veterinary  
98 Medicine Ethics and Welfare Committee No. CR695 (2023) & CR496 (2021). DNA was extracted  
99 from both blood and buccal swabs using QIAamp DNA Blood Mini or Midi Kits (Qiagen, Manchester,  
100 UK). This study was performed in accordance with the ARVO Statement for Use of Animals in Research.  
101 Dogs were designated as RVED cases or controls following examination by board-certified veterinary  
102 ophthalmologists. The inclusion criteria for cases and controls were as follows:

103 1. Controls: ECS aged 6 years or older with no evidence of retinopathy on ophthalmoscopy (Fig.1).

104 2. Cases:

105 a) RVED-affected cases. ECS presenting with owner-perceived visual deficits,  
106 ophthalmoscopic signs consistent with RVED and plasma  $\alpha$ -Toc concentrations  
107  $<20\mu\text{mol/l}$  (Fig.2 and Fig.3). One case was reported to be an English Cocker  
108 Spaniel/Cavalier King Charles Spaniel (ECS/CKCS) cross.

109 b) RVED-suspected cases. ECS presenting with owner-perceived visual deficits,  
110 ophthalmoscopic signs consistent with RVED but for which plasma  $\alpha$ -Toc  
111 concentrations were unavailable (Fig.2 and Fig.3).

## 112 **Ophthalmoscopy**

113 Control and case status was established following ophthalmoscopy by a board-certified veterinary  
114 ophthalmologist. Following pharmacological mydriasis with 1% tropicamide, each eye of each dog was  
115 examined with both indirect and direct ophthalmoscopy (equipment varied between  
116 ophthalmologists). Control dogs had no evidence of retinopathy (Fig. 1) and cases had signs consistent  
117 with RVED based on previous published clinical descriptions of the disease (McLellan et al. 2003).  
118 Affected dogs are recognised clinically by the appearance of light brown pigment spots (Fig.2), and  
119 subsequently patches, in the tapetal fundus. Degeneration of the neurosensory retina results in  
120 associated areas of increased tapetal reflectivity (Fig.3).

## 121 **Genome-wide association study (GWAS)**

122 Genotyping of 20 ECS RVED cases and 30 ECS controls was carried out using the Illumina® CanineHD  
123 230k array. The GWAS data were analysed for association using PLINK version 1.9 (Purcell et al. 2007).  
124 Quality control of data included the exclusion of SNPs with a minor allele frequency of  $<5\%$  and missing  
125 genotype calls of  $>10\%$  and the sample call rate for individuals was  $>99.7\%$ . A multi-dimensional

126 scaling (MDS) plot was generated using PLINK to assess for the presence of population stratification.  
127 A plot of negative log (base 10) P-values was performed. A p-value of 0.05 after correction for multiple  
128 testing using the Bonferroni correction was the threshold for statistical significance. A test of SNPs in  
129 linkage disequilibrium ( $r^2$ ) with the top SNP from the GWAS was carried out in PLINK.

130

### 131 **Whole-genome sequencing**

132 Two ECS cases were selected for whole genome sequencing (WGS). Sequencing was outsourced to  
133 Edinburgh Genomics, UK where a TruSeq Nano 150bp paired-end library was prepared and sequenced  
134 on the Illumina HiSeq X platform, generating approximately 30X genome coverage. Read data were  
135 aligned to the CanFam4 UU Cfam GSD 1.0 reference genome using BWA-MEM v0.7 (Li and Durbin  
136 2009). Base quality score recalibration, indel realignment and duplicate removal was performed using  
137 the Genome Analysis Toolkit (GATK) v4.2 according to GATK Best Practices recommendations  
138 (McKenna et al. 2010; Poplin et al. 2018). SNP/INDEL discovery was performed using GATK  
139 HaplotypeCaller (McKenna et al. 2010) and then loaded into a GenomicsDB. Joint variant calling was  
140 performed across 309 samples, including the two ECS cases, and the resulting variant calls filtered  
141 using standard hard-filtering parameters. The filtered variants were annotated and functional effects  
142 predicted using SnpEff v5.1 (Cingolani et al. 2012) and visualised in the Integrative Genomics Viewer  
143 (IGV) software (Robinson et al. 2022; Robinson et al. 2011).

### 144 **WGS variant filtering**

145 The first stage of variant filtering was performed using WGS of 307 dogs comprising 109 breeds and  
146 two cross breeds, via an in-house pipeline that scores variants based on the predicted effect on the  
147 protein. The 307 WGS consist of dogs with varying phenotypes excluding RVED, therefore all 307 dogs  
148 acted as controls. Pedigree analysis of RVED cases indicated a recessive mode of inheritance for the  
149 disease and therefore a criterion of the variant filtering was that both cases had to be homozygous

150 for an alternate allele and controls either heterozygous or homozygous for the reference allele.  
151 Variants retained after the first stage of filtering, with the highest effect score and homozygous in  
152 both cases were then further filtered against a Variant Call Format (VCF) file containing 1987 WGS  
153 consisting of 1611 dogs (321 breeds), 309 village dogs, 63 wolves, and four coyotes curated by the  
154 Dog10K Consortium (Meadows et al. 2023).

155

156 **Genotyping of WGS filtered variant by Sanger sequencing and Amplified Fragment Length**  
157 **Polymorphism (AFLP)**

158

159 After the exclusion of common (present in multiple canine breeds) variants a single variant remained  
160 after filtering which was homozygous in both RVED-affected ECS. The variant was a deletion located  
161 in exon 1 of *TTPA*. The variant was verified initially by Sanger sequencing of two cases and two  
162 controls. PCR products were amplified using HotstarTaq DNA Polymerase (Qiagen), 1.5mM 60:40  
163 d7GTP:GTP dNTP mix and Q Solution (Qiagen). Cycling conditions were: 98°C for 15 mins; 35 cycles at  
164 98°C for 30s; 59°C for 30s; 72°C for 30s; and 72°C for 5mins. Amplified products were sequenced in  
165 both directions at Source Bioscience, Cambridge, UK. Sequence traces were analysed using the Staden  
166 software package (Staden et al. 2000). Further variant validation was carried out by AFLP in 30 cases  
167 and 43 controls. PCR products were amplified using HotstarTaq DNA Polymerase (Qiagen), 1.5mM  
168 60:40 d7GTP:GTP dNTP mix, Q Solution (Qiagen), a FAM fluoresced tailed forward primer (S2 Table).  
169 Cycling conditions were: 98°C for 15 mins; 35 cycles at 95°C for 30s; 59°C for 30s; 72°C for 1min and  
170 then 8 cycles at 94°C for 30s; 50°C for 30s, 72°C for 1min and then 72°C for 30mins. Primers were  
171 designed using Primer3 (Untergasser et al. 2012) (S2 Table) to flank the deletion. Amplified products  
172 were outsourced to the Department of Biochemistry, University of Cambridge, UK, for AFLP using an  
173 ABI 3130xl DNA Analyzer (Applied Biosystems). Fragment length analysis was then carried out using  
174 Genemarker v.3.0.1 (Softgenetics LLC, USA ).

175

176 A further 186 ECS were genotyped by AFLP as above to ascertain the variant frequency.

177

## 178 **Results**

### 179 **RVED Cases**

180 A total of 21 RVED-affected and 9 RVED-suspected ECS samples were analysed for this study (S1 Table).

181 Five-suspected RVED cases reportedly also had low circulating plasma  $\alpha$ -Toc concentrations but

182 laboratory results were unavailable to confirm this. The mean age at diagnosis of the cases was 5.28yrs

183 and the median age, 5yrs. The mean  $\alpha$ -Toc plasma concentration where levels were provided ( $n=21$ )

184 was 5.65 $\mu$ mol/l and the median 3.3 $\mu$ mol/l. Two cases aged 3.17 and 6.67yrs at diagnosis showed

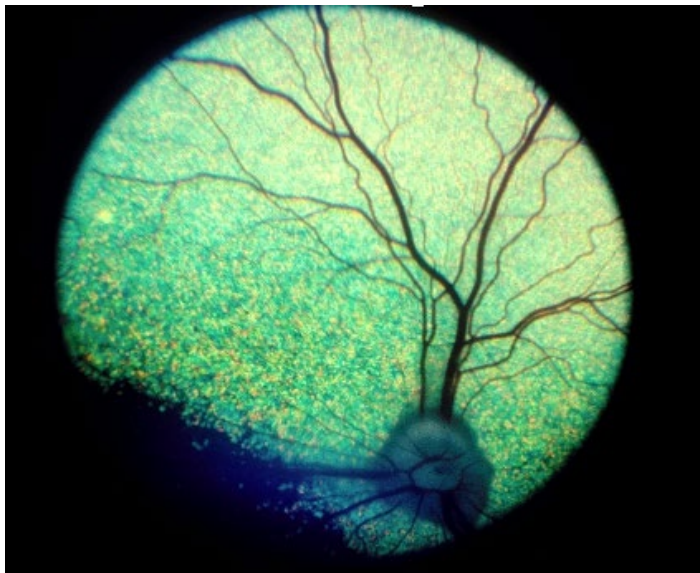
185 neurological clinical signs of hindlimb proprioceptive deficits with the latter also observed to have

186 hindlimb ataxia. One case aged 3yrs suffered from seizures. Facial nerve paresis was reported in a 7yr

187 old ECS case. Pedigree analysis indicated that the disease segregates with a recessive mode of

188 inheritance. Figure 1 shows a fundus photograph of one of the control dogs. Figures 2 and 3 illustrate

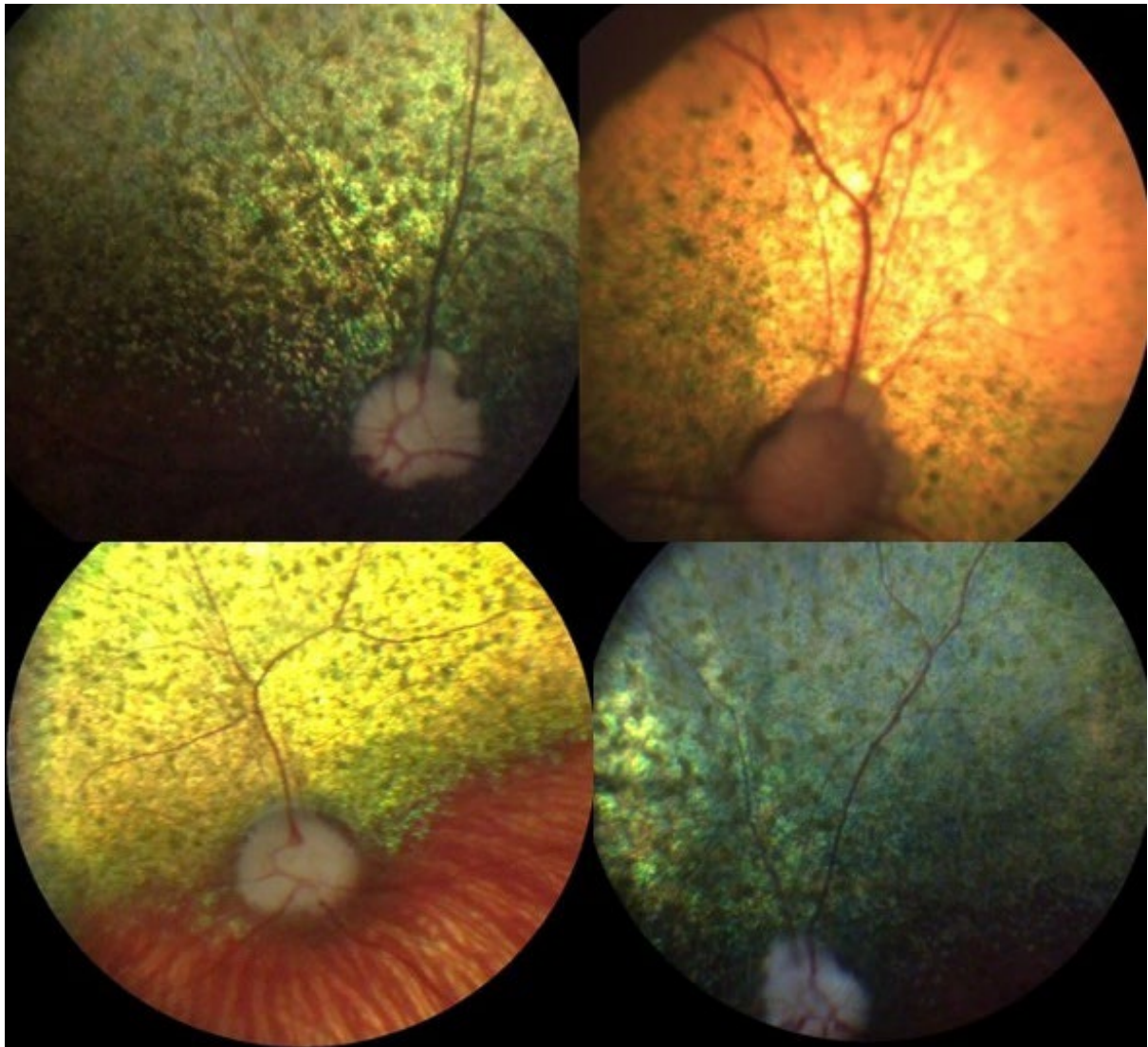
189 fundic disease presentation of RVED at early and late stages of the disease respectively.



190

191 **Figure 1.** Fundus photograph of one RVED-unaffected English Cocker Spaniel included in this study

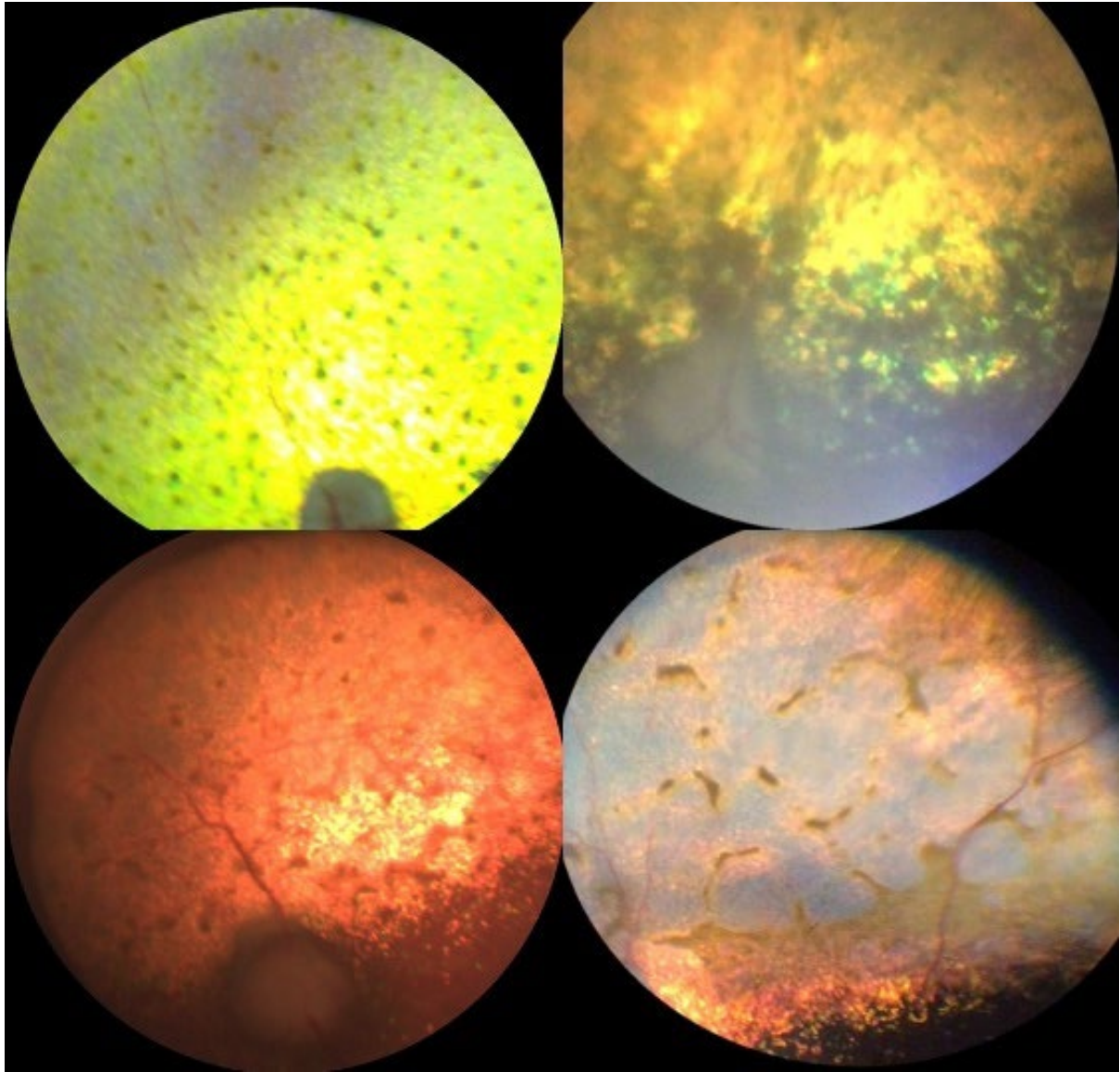
192 (control).



194

195 **Figure 2.** Fundus photographs of four RVED-affected English Cocker Spaniels with early retinopathy  
196 with vitamin E deficiency included in this study (cases). Multifocal grey-brown spots are present within  
197 the tapetal fundus of each dog representing lipofuscin granule deposition within the retinal pigment  
198 epithelium.

199



200

201

202 **Figure 3.** Fundus photographs of four RVED-affected English Cocker Spaniels with advanced  
203 retinopathy with vitamin E deficiency included in this study (cases). All cases show significant retinal  
204 degeneration characterised by retinal vascular attenuation and tapetal hyperreflectivity.

205

### 206 **Genome-wide association study**

207 After quality control, the genome-wide association analysis was carried out using genotyping data  
208 containing 127,557 SNPs, 18 cases and 30 controls. A strong statistical signal was observed on  
209 chromosome 29 ( $P_{\text{raw}} 1.909 \times 10^{-17}$ ) exceeding the Bonferroni threshold  $-\log_{10}$  p-value of 6.41 (Fig.4). A

210 second signal exceeding the Bonferroni threshold occurs on chromosome 13 ( $P_{\text{raw}}7.42 \times 10^{-8}$ ). A region  
211 containing SNPs that were in linkage disequilibrium with the top SNP (BICF2P546283) (i.e. with an  $r^2$   
212 value  $>0.80$ ) spanned a region of approximately 4.7Mb from SNP chr29\_13257379 to BICF2S23527829  
213 (chr29:13257379 – 16318933 based on CanFam3.1).

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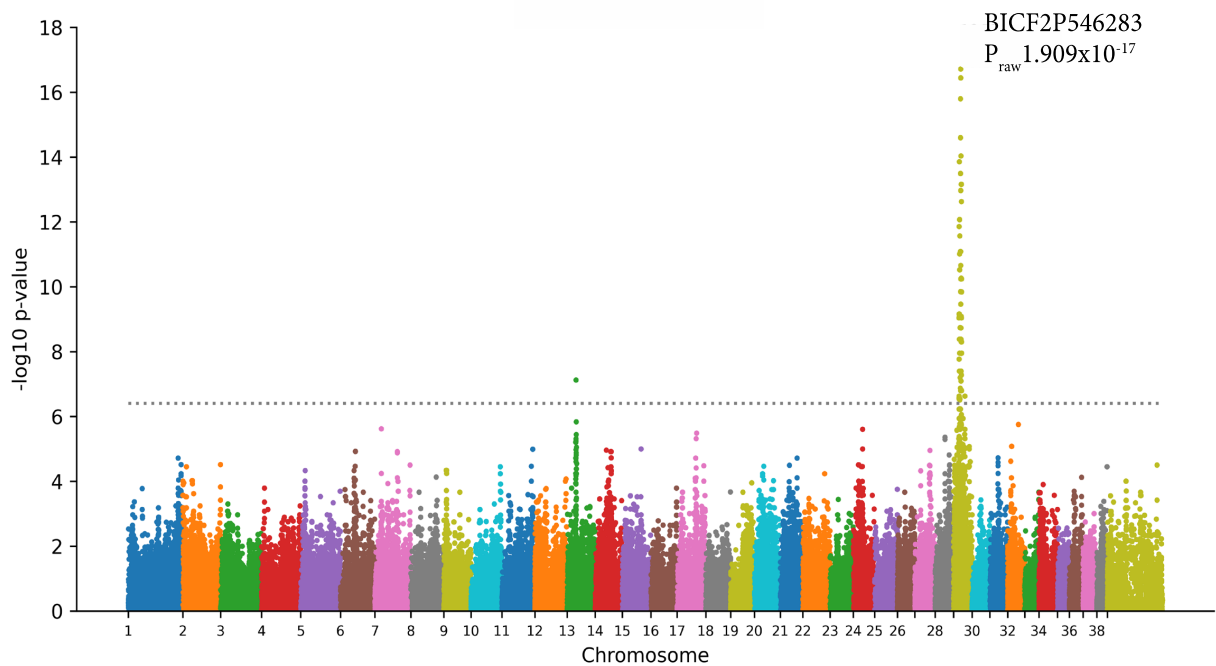
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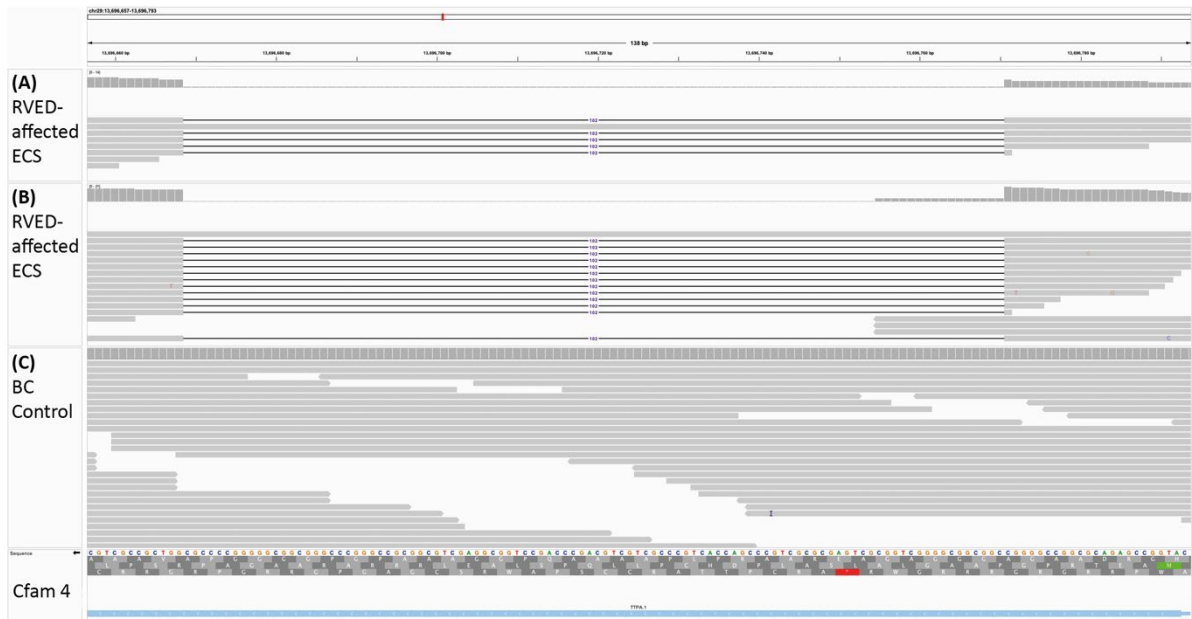
225 **Figure 4. Genome-wide association analysis of RVED in English Cocker Spaniels**

226 Manhattan plot of association of RVED in ECS is shown. Genome-wide association significance is  
227 determined by Bonferroni correction shown by the dotted line. The top SNP is shown with the  $\text{Log}_{10}$   
228  $P_{\text{raw}}$  value. All GWAS SNPs are based on the CanFam3.1 reference genome.

229

230 Twenty-two variants with the highest effect on the protein were homozygous in the two ECS RVED-  
231 affected WGS. Only one variant was private to the cases after filtering against our in-house 307 WGS  
232 and the Dog10K VCF (Meadows et al. 2023). The variant was a 102bp deletion located in exon 1 of  
233 *TTPA*, the gene that encodes the alpha-tocopherol transfer protein (Fig.5). The deletion truncates the  
234 protein by 34 amino acids but remains in frame (Fig.6).

235



236

237 **Figure 5. WGS reads in IGV of RVED-affected ECS and a control RVED-unaffected Border Collie dog**

238 WGS reads are based on the canine CanFam4 UU Cfam GSD 1.0 reference genome. (A) and (B)

239 illustrate local alignments generated using GATK HaplotypeCaller using the genomic co-ordinates

240 produced by the VCF of the two ECS RVED cases (Poplin et al. 2018). The 102bp deletion is illustrated

241 in (A) and (B) with black lines joining alignments annotated with deletion size . Reads in (A) and (B)

242 without the 102bp annotation are representative of artificial haplotypes created by HaplotypeCaller

243 and based on the reference sequence. Reads shown in (C) are from an RVED-unaffected Border Collie

244 control WGS. The 102 bp deletion is located in exon 1 of *TTPA* (chr29:13,696,668-13,696,771).

245

246

247

248 **Figure 6. Sequence of 102bp deletion identified in exon 1 of *TTPA***

249 The 102bp nucleotide deletion is shown in red with the amino acid translation above. The  
250 deletion remains in frame. It commences at p.P8 in the last two nucleotides of the codon and  
251 ends with the first nucleotide of p.R42. The nucleotides flanking the deletion (underlined)  
252 result in a missense amino acid change from P>R at p.8 and the sequence then continues as  
253 per the wild-type sequence from position p.43.

254  
255

256 **Potential pathogenicity of c.23\_124del *TTPA* variant**

257

258 To ascertain the potential pathogenicity of the c.23\_124del *TTPA* variant we analysed the  
259 level of conservation of deleted amino acids and searched for annotated functional domains  
260 within the protein in the human, mouse and dog (Fig.7). We also evaluated the effects the  
261 variant may have on the protein structure using Alphafold (Fig.8 and 9) (Jumper et al. 2021).

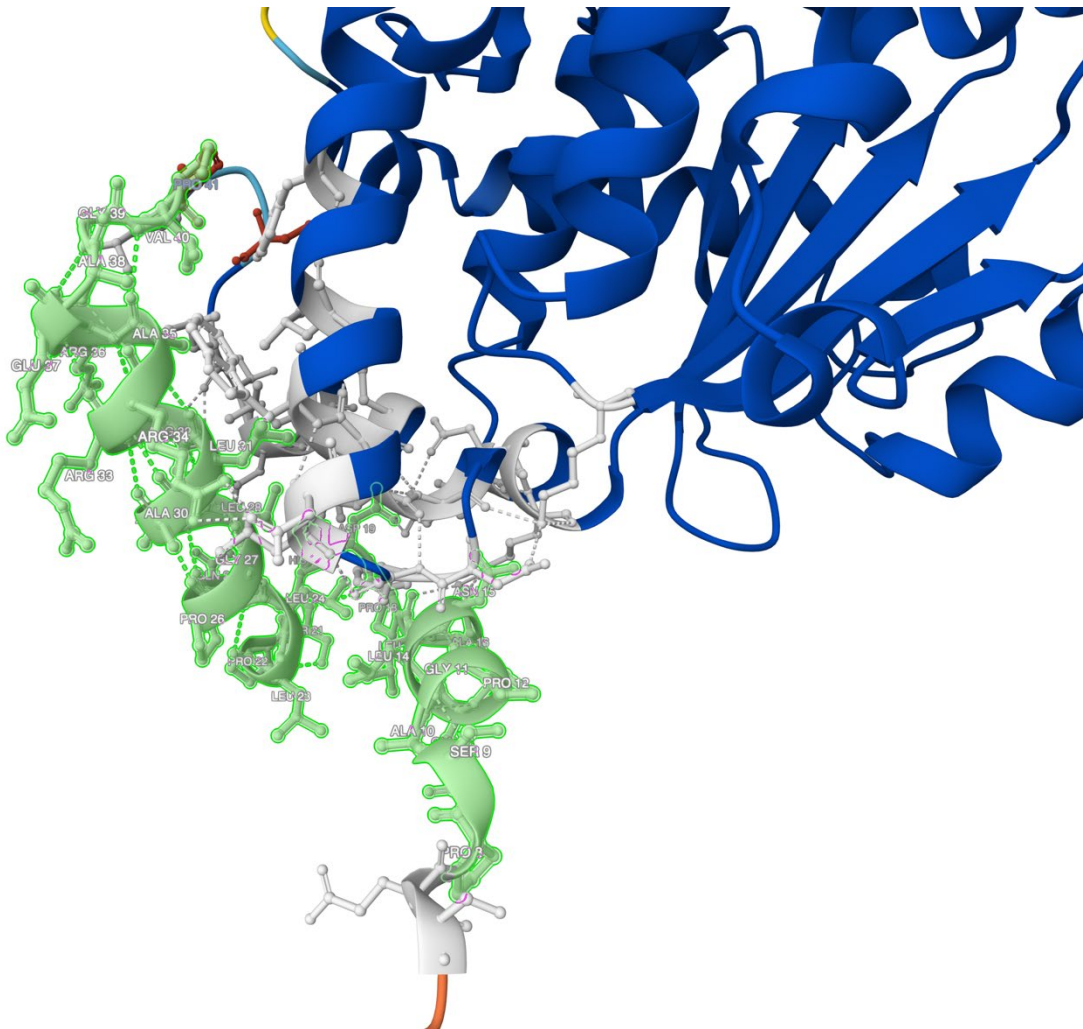
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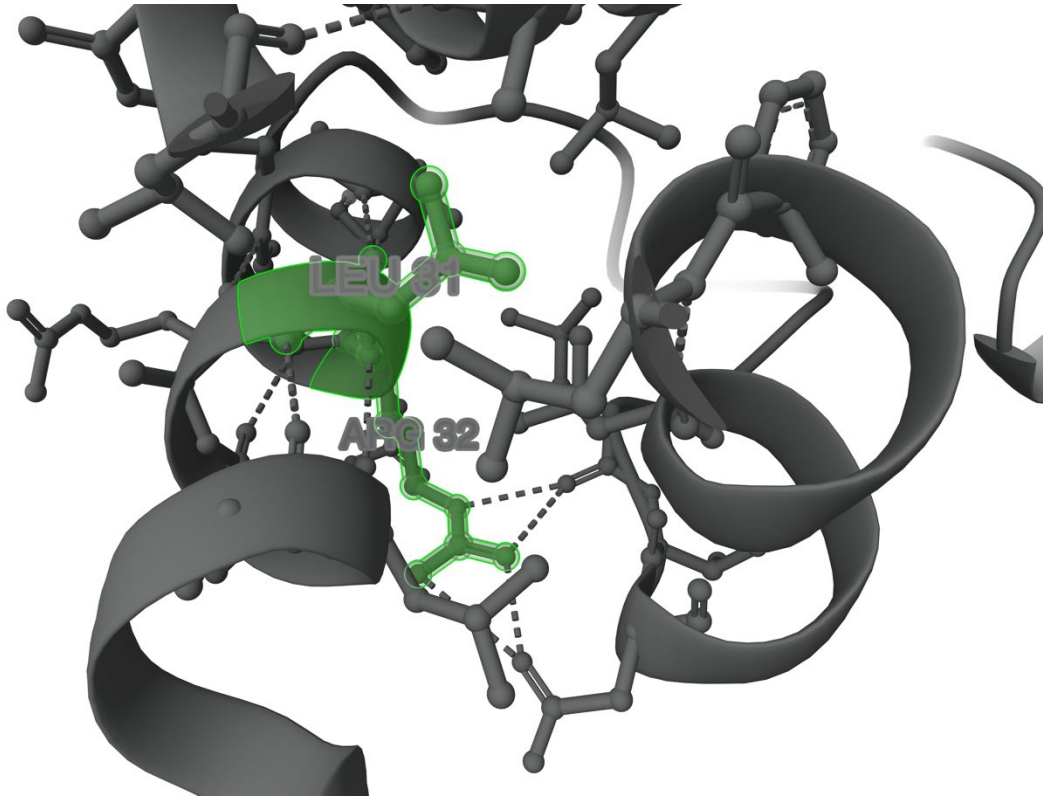
264 **Figure 7. Human, canine and mouse  $\alpha$ -TTP protein alignment**

265 The  $\alpha$ -TTP amino acid alignment between human (NP\_000361.1), mouse (NP\_056582.1) and dog  
266 (XP\_038297173.1) shows predicted functional domains of the protein. Amino acids are coloured in  
267 red indicating this is a highly conserved region (based on the relative entropy threshold of the  
268 residue). The deletion removes amino acids from position p.8 to p.42. The CRAL-TRIO-N lipid binding  
269 domain in the mouse and human would be affected in addition to the Disordered domain in the  
270 human. The CRAL-TRIO domain commences at p.47 in the dog and is therefore not predicted to be  
271 affected. The alignment was carried out using the National Center for Biotechnology Information

272 Constraint-based Multiple Alignment Tool version 1.25.1 and coloured using the 'Conservation'  
273 method (Papadopoulos and Agarwala 2007).  
274



275  
276 **Figure 8. AlphaFold prediction of the 3-D  $\alpha$ -TTP protein structure in the human**  
277 The graphic shown in Fig.8 is the predicted  $\alpha$ -TTP protein configuration in the human generated by  
278 AlphaFold (Jumper et al. 2021; Varadi et al. 2021; Varadi et al. 2023). The per-residue model  
279 confidence score is 'low' (pLDDT 0-70) from p.1 to p.10, 'high' from p.11 to p.13 (pLDDT 70-90) and  
280 'very-high' (pLDDT>90) from position p.14. The region annotated by amino acid position and shown  
281 in green is the 34 amino acids deleted from the ECS RVED cases. The interactions of deleted amino  
282 acids with surrounding amino acids are shown in grey and illustrate their relationship with the  
283 adjacent  $\alpha$ -helix. Dotted lines represent hydrogen bonds between amino acids.



284

285 **Figure 9. AlphaFold 3-D human  $\alpha$ -TTP protein centred on p.L31 and p.R32**

286 Protein positions p.L31 and p.R32 are shown highlighted in green. AlphaFold predicts that in the  
287 human, any amino acid alteration at these locations would be pathogenic (Minton 2023).

288

289 ***TTPA* variant validation**

290 The c.23\_124del variant in *TTPA* was validated in a cohort of 30 ECS RVED cases and 43 ECS controls  
291 using AFLP. The variant segregated correctly with the disease with all cases homozygous for the 102bp  
292 deletion. The results are shown in Table 1. The variant frequency among the clinically unaffected  
293 controls (all heterozygous for the deletion) was 0.09. Further genotyping by AFLP was carried out in a  
294 cohort of 186 ECS of unknown health status to ascertain the frequency of the variant (Table 2). The  
295 variant frequency in this cohort was 0.11 which is comparable to the clinically unaffected control  
296 cohort of ECS (Table 1)

297

298 **Table 1. Results of amplified fragment length polymorphism analysis of cases and controls for the**  
 299 ***TTPA* deletion**

<b>Disease Status</b>	<b>Homozygous deletion del/del</b>	<b>Heterozygous deletion WT/del</b>	<b>Homozygous wild-type WT/WT</b>	<b>Total</b>
<b>RVED Cases</b>	30	0	0	<b>30</b>
<b>Controls</b>	0	8	35	<b>43</b>

300

301 **Table 2. Results of amplified fragment length polymorphism for the *TTPA* deletion in 186 ECS**  
 302 **randomly selected from an archived collection**

	<b>Homozygous deletion del/del</b>	<b>Heterozygous deletion WT/del</b>	<b>Homozygous wild-type WT/WT</b>	<b>Total number of ECS</b>
Number of ECS	7	26	153	<b>186</b>

303

304 Seven ECS were homozygous for the variant and were subsequently followed up to ascertain the  
 305 health status of each dog. The results of the follow-up are shown in Table 3.

306

307 **Table 3. Health status of dogs homozygous for the 102bp *TTPA* deletion after AFLP analysis**

<b>Dog Number</b>	<b>Health Status</b>	<b>Age submitted</b>
<b>1</b>	Suspected neuronal ceroid-lipofuscinoses (NCL) , blind	~ 3 yrs
<b>2</b>	Anterior subcapsular cataract (left eye)	0.75 yrs
<b>3</b>	Suspected progressive retinal atrophy (veterinarian submission)	6.5 yrs
<b>4</b>	Early signs of retinal degeneration	3 yrs

5	Unknown status	N/A
6	Owner submitted as 'clear' of disease	0.2 yrs
7	Owner submitted as 'clear' of disease	3.5 yrs

308

309 **Discussion**

310 In this study we employed a combined approach of GWAS and WGS to identify a 102bp  
311 deletion in *TTPA* that is associated with RVED in the ECS. The variant was identified by WGS pipeline  
312 analysis of two cases aligned to the canine reference genome, CanFam4. The region identified in the  
313 GWAS (Fig.4) strongly suggested an association with RVED on chromosome 29. The SNPs in linkage  
314 disequilibrium with the top SNP spanned a region that commenced at chr29: 13257379 which is within  
315 *YTHDF3*, upstream of *TTPA*. It was interesting to note that the top SNP was not located within *TTPA*  
316 itself. However, the SNPs on the Illumina® CanineHD 230k array are all based on CanFam3.1 co-  
317 ordinates, a version of the reference genome in which *TTPA* is incorrectly annotated. There was an  
318 additional SNP that exceeded the Bonferroni threshold located on chromosome 13. Homozygosity  
319 mapping of RVED cases and controls did not reveal potential blocks of homozygosity present in all  
320 cases. Two RVED-affected ECS however, did have a homozygous region on chromosome 13  
321 surrounding the most associated SNP (data not shown). Visual interrogation of WGS reads of the two  
322 RVED-affected ECS in IGV in conjunction with WGS pipeline analysis did not reveal anything of  
323 significance in this region. We postulate, therefore, that the SNP on location 13 is specific to two  
324 individual cases and not related to RVED.

325 *TTPA* is an excellent candidate gene for RVED because mutations in this gene have been  
326 reported to cause a similar phenotype in humans. In humans, *TTPA* mutations are responsible for  
327 AVED, a disorder previously known as familial isolated vitamin E deficiency (FIVE) (Alex et al. 2000;  
328 Bouhlal et al. 2008; Cellini et al. 2002; Di Donato et al. 2010; El Euch-Fayache et al. 2014; Elkamil et al.  
329 2015; Hoshino et al. 1999; Mariotti et al. 2004; Schuelke 1993; Zea Vera et al. 2021; Zhang et al. 2022).  
330 Initial signs of AVED include progressive ataxia, clumsiness of the hands, loss of proprioception and

331 areflexia (Schuelke 1993). Retinitis pigmentosa also appears fairly common in AVED patients  
332 (Abramowicz et al. 2024; Iwasa et al. 2014; Pang et al. 2001; Shimohata et al. 1998; Yokota et al. 1996;  
333 Yokota et al. 1997; Yokota et al. 2000). In our study, all affected ECS had pigmentary retinopathy  
334 although neurological disturbance was only reported in three dogs. A fourth dog was reported to have  
335 facial nerve paresis, however, it is uncertain as to whether this is associated with low plasma vitamin  
336 E (Cameron et al. 2007). It is likely that ataxia is more common in dogs with RVED than our study may  
337 suggest. A previous study of RVED in the ECS in which all dogs were examined physically,  
338 ophthalmologically and neurologically reported neurological dysfunction to be common (McLellan et  
339 al. 2003). Eleven of fifteen dogs (73%) showed signs including ataxia, proprioceptive deficits, abnormal  
340 spinal reflexes and muscle weakness. Sample collection in our study was biased. All samples were  
341 recruited from veterinary ophthalmologists who were presented cases, following owner perception  
342 of visual deficits in their dogs. Had samples also been recruited from veterinary neurologists, more  
343 cases of RVED with ataxia may have been identified. It is possible that dog 1 (Table 3) that was  
344 suspected to be a neuronal ceroid-lipofuscinoses case but that was in fact homozygous for the *TTPA*  
345 c.23\_124del variant is an example of such a case. Furthermore, although retinopathy was the most  
346 obvious presenting clinical sign in the affected dogs, no dogs underwent thorough neurological  
347 examination and so signs of subtle neurological dysfunction may have been missed.

348 *TTPA* encodes the  $\alpha$ -tocopherol transfer protein ( $\alpha$ -TTP) which is the only known protein to  
349 specifically bind  $\alpha$ -Toc – the most abundant and biologically active form of vitamin E in higher animals  
350 (Arai and Kono 2021).  $\alpha$ -TTP is highly expressed in the liver where  $\alpha$ -TTP selects  $\alpha$ -Toc taken up via  
351 plasma lipoproteins and promotes its secretion to circulating lipoproteins (Traber et al. 1992; Traber  
352 and Kayden 1989; Traber et al. 1990). Thus,  $\alpha$ -TTP is a major determinant of plasma  $\alpha$ -Toc  
353 concentrations. Although,  $\alpha$ -TTP is highly expressed and has an important function in the liver, it is  
354 also expressed in the lung, spleen, uterus, brain and retina (Hosomi et al. 1998; Kaempf-Rotzoll et al.  
355 2002; Shichiri et al. 2012).  $\alpha$ -TTP mRNA is detected predominantly in the Purkinje layer of the  
356 cerebellar cortex and, in AVED patients, loss of  $\alpha$ -TTP causes severe damage to Purkinje cells in the

357 brain (Hosomi et al. 1998; Larnaout et al. 1997; Ulatowski et al. 2014).  $\alpha$ -TTP is expressed in Müller  
358 cells of the retina which may facilitate the transport of  $\alpha$ -Toc from the blood capillaries to  
359 photoreceptor neurons (Shichiri et al. 2012). The photoreceptor outer segment membrane contains  
360 unusually high amounts of polyunsaturated fatty acids, which makes the membrane more susceptible  
361 to oxidation (Neuringer et al. 1988). In addition to its role in preventing lipid peroxidation,  $\alpha$ -Toc may  
362 also protect oxidation of vitamin A which is essential to the visual process (Robison et al. 1979; 1980).  
363  $\alpha$ -Toc may also have a role in maintaining photoreceptor membrane fluidity which is necessary for  
364 the normal movement of rhodopsin molecules during phototransduction (Goss-Sampson et al. 1991;  
365 Moran et al. 1987).

366 In both dogs and humans, *TTPA* comprises 5 exons which encode a 278 amino acid translated  
367 product. To date, over 20 deleterious variants in *TTPA* have been reported to be associated with AVED  
368 in humans. Variants have been found in each of the five exons (Amiel et al. 1995; Cavalier et al. 1998;  
369 Di Donato et al. 2010; Gotoda et al. 1995; Hoshino et al. 1999; Ouahchi et al. 1995; Shimohata et al.  
370 1998; Usuki and Maruyama 2000; Yokota et al. 1996; Yokota et al. 1997; Yokota et al. 2000). Variant  
371 type broadly correlates with both the age of onset and severity of clinical signs with truncations,  
372 frame-shift variants and non-conserved substitutions resulting in more severe and early onset forms  
373 of AVED, in association with dramatic reductions in plasma  $\alpha$ -Toc concentrations (Amiel et al. 1995;  
374 Hentati et al. 1996; Krendel et al. 1987; Mariotti et al. 2004; Roubertie et al. 2003). To the authors'  
375 knowledge, the variant we report is the only naturally occurring mutation in *TTPA* in a non-human  
376 species. The mutation is a 102bp deletion in exon 1 of *TTPA* which is predicted to result in a protein  
377 that is truncated by 34 amino acids and which presumably leads to loss of function, accounting for the  
378 severe reduction in plasma  $\alpha$ -Toc concentrations and retinopathy, reported to occur in ECS with RVED.  
379 The deletion was private to the RVED cases after WGS filtering, however it was subsequently identified  
380 in the UCSC genome browser (CanFam4) (Nassar et al. 2023). It was annotated whilst creating the  
381 structural variation track as part of the GSD\_1.0/CanFam 4 reference assembly (Wang et al. 2021).  
382 The track was created from 10x sequencing data of 27 dogs of 19 breeds. Further investigation found

383 that of the 27 dogs, one ECS was homozygous for the variant. Follow-up of the dog revealed that it  
384 had unilateral glaucoma but funduscopy was not performed and so RVED status is unknown. One  
385 RVED case was reported by the owner to be an ECS/Cavalier King Charles Spaniel cross. The dog was  
386 homozygous for the *TTPA* deletion variant. Genetic verification of the cross was not provided and  
387 therefore we postulate that as the variant was not detected in other dog breeds that this RVED case  
388 was not an F1 cross (ECS x Cavalier King Charles Spaniel) but rather an F2 cross with ECS on both the  
389 sire and dam's sides of its pedigree.

390         The potential effects that the c.23\_124del variant may exert on the structure of the  $\alpha$ -TTP  
391 protein in the human is shown in Fig.8. The first three  $\alpha$ -helices would be deleted and thus would not  
392 provide structural support to surrounding structures. We can infer that this occurrence is analogous  
393 in the canine TTPA protein as amino acids in this region are highly conserved between the two species  
394 (Fig.7). Two amino acids located at p.L31 and p.R32 are highlighted in Fig.9, both of which are highly  
395 conserved in mammals (S1 Fig.). AlphaFold predicts a change of amino acid (any amino acid) at these  
396 locations would be deleterious (Fig.9). Amino acid p.L31 is maintaining the first  $\alpha$ -helix structure and  
397 amino acid p.R32 forms hydrogen bonds with p.L47 and p.D49. AlphaFold computes an average  
398 missense pathogenicity of any amino acid alteration at these locations to be 0.797 and 0.710  
399 respectively and are therefore predicted to be pathogenic (Minton 2023). We postulate, therefore,  
400 that deletion of these amino acids would also be pathogenic in the ECS and disrupt the protein  
401 structure. In the canine protein (Fig.7) conserved domains are not predicted to be affected by the  
402 c.23\_124del variant. However, in both the human and mouse the CRAL-TRIO N domain would be  
403 impacted. This is an important domain that facilitates interactions with bound lipid head groups (Li et  
404 al. 2023). Furthermore, in the human protein, the intrinsically disordered region would be removed  
405 as a result of the c.23\_124del variant. Disordered regions are malleable and thus do not conform to a  
406 rigid 3-D structure, they are important for biological processes such as cell signalling and subcellular  
407 organization (Holehouse and Kragelund 2024). This domain is not annotated in the dog but it is

408 unknown as to whether this is a consequence of a lack of functional and computation analysis of the  
409 canine protein or whether it is truly absent.

410 RVED-affected dogs have been reported to have mean a plasma  $\alpha$ -Toc concentration of  
411 3.78 $\mu$ mol/l compared to 67.11 $\mu$ mol/l in unaffected dogs (McLellan et al. 2002). This is consistent with  
412 the median concentration of 3.3 $\mu$ mol/l in the cases in our study, and in which the mean  $\alpha$ -Toc was  
413 5.65 $\mu$ mol/l. AVED is treated with vitamin E supplementation (Meydani et al. 1998). Treatment results  
414 in cessation of progression of signs of neurological dysfunction in most patients and improvement of  
415 signs in some (Gohil and Azzi 2008; Kohlschütter et al. 2020; Yokota et al. 1996). Favourable response  
416 to treatment relates to severity and duration of clinical signs before treatment is implemented.  
417 Treatment of presymptomatic individuals has also prevented development of AVED further underlying  
418 the importance of prompt treatment and also early identification of those at risk patients through  
419 molecular genetic testing (Schuelke 1993). In dogs with RVED, oral supplementation with vitamin E  
420 restored plasma  $\alpha$ -Toc concentrations to within the normal reported range and appeared to halt  
421 progression of neurological disease, improve neurological signs and exercise tolerance but not lead to  
422 resolution of pre-existing ocular signs (McLellan and Bedford 2012; McLellan et al. 2003; McLellan et  
423 al. 2002). To date, it has not been possible to identify affected dogs until the onset of consistent clinical  
424 signs in association with severe reduction in plasma  $\alpha$ -Toc concentrations. Now that a variant  
425 associated with RVED has been identified, a molecular DNA test can be developed which will both  
426 allow identification of presymptomatic individuals for therapeutic intervention along with those that  
427 carry the mutation. With appropriate use of DNA testing, breeders will be able to eradicate RVED from  
428 the breed efficiently. The ECS is a popular breed of dog in the United Kingdom with approximately  
429 26,000 dogs being registered with the Kennel Club each year (Kennel Club data). Considering an  
430 estimated variant frequency of 0.09 and assuming random segregation of the variant within the  
431 population, this would equate to approximately 210 Kennel Club-registered ECS being homozygous  
432 for the mutation and thus affected with RVED each year. This itself is likely to be a gross underestimate

433 of the total number of ECS affected by RVED in the United Kingdom, as only a minority of ECS are  
434 registered with the Kennel Club.

435 In conclusion, we have identified a deletion in TTPA that is associated with RVED in ECS. This  
436 appears to be the only spontaneously occurring mutation in TTPA in a non-human species and, as in  
437 humans with AVED, occurs as an autosomal recessive trait.

438

#### 439 **Data Availability Statement**

440 Whole genome sequencing data for this study have been deposited in the European  
441 Nucleotide Archive (ENA) at EMBL-EBI under accession numbers: PRJEB79956 and PRJEB36029.  
442 BioSample accessions for the two RVED cases are SAMEA7189992 and SAMEA7190009.

443

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449

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455

#### 456 **Conflicts of Interest**

457 Cathryn Mellersh manages a DNA testing service that is currently offering a commercial DNA  
458 test for this variant. The authors declare that no other competing interests exist. The funders had no

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461

462

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