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## Phenotypic variability in patients with retinal dystrophies due to mutations in CRB1

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1 TITLE: Phenotypic variability in patients with retinal dystrophies due to mutations in *CRB1*

2

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25

26

27 **ABSTRACT:**

28 **Objectives:** To identify CRB1 mutations in a large cohort of patients with recessive retinal  
29 dystrophies and to document the retinal phenotype and visual prognosis.

30

31 **Design:** A hospital based cross sectional study of children and adults with recessive retinal  
32 dystrophies.

33

34 **Participants:** Three hundred and six patients with Leber's congenital amaurosis (LCA),  
35 early onset childhood retinal dystrophy (EORD), or juvenile onset retinitis pigmentosa (arRP)  
36 were recruited to the study and gave blood samples for molecular genetic analysis

37

38 **Methods:** A detailed clinical examination was performed including: LogMAR visual acuity,  
39 refraction, Goldmann visual fields, slit-lamp biomicroscopy, fundus photography,  
40 autofluorescence imaging and optical coherence tomography. The results of  
41 electrophysiology testing were available in all patients. DNA was obtained for molecular  
42 genetic analysis. Initial screening for mutations was performed using the LCA chip (Asper  
43 Ophthalmics). Patients who had one or more CRB1 mutations identified on the chip, and  
44 other patients whose phenotype suggested a CRB1 genotype, underwent direct sequencing. In  
45 addition, consanguineous families segregating recessive RP underwent a whole genome scan  
46 using Affymetrix gene chips (Affymetrix UK Ltd) and affected family members showing  
47 linkage to the RP12 locus underwent sequencing of the CRB1 gene.

48

49 **Main outcome measures:** Identification of patients with mutations in CRB1 and detailed  
50 documentation of the clinical phenotype

51

52 **Results:** Mutations in CRB1, including 17 novel mutations, were identified in 41 patients  
53 from 32 families. We identified both disease mutations in 34 patients from 26 families and  
54 these patients underwent detailed phenotyping. Common phenotypic features included  
55 hypermetropic refractive error; nummular pigmentation at the level of the RPE; and increased  
56 retinal thickness on OCT. Most patients had a clinical and electrophysiological phenotype  
57 consistent with a diagnosis of LCA or rod-cone dystrophy but three patients had ERG  
58 evidence of cone-rod degeneration. A minority of patients developed peripheral retinal  
59 telangiectasia, which in some cases led to seclusio pupillae and angle closure glaucoma.

60

61 **Conclusion:** Mutations in CRB1 are associated with a range of recessively inherited retinal  
62 dystrophies including LCA, childhood and juvenile onset rod-cone, and cone-rod dystrophies.  
63 Although the phenotype is usually severe, in milder cases there is a window of opportunity  
64 for therapeutic intervention in early childhood.

65

66 Retinitis Pigmentosa (RP) is a generic term used to describe a group of inherited retinal  
67 degenerations that lead to death of rod and cone photoreceptors. RP has a prevalence of about  
68 1 in 3-4000. It is clinically and genetically heterogeneous with autosomal dominant,  
69 autosomal recessive and X-linked inheritance being described.[1] Even amongst these  
70 subtypes there is considerable genetic heterogeneity with over 100 genes currently known  
71 (<http://www.sph.uth.tmc.edu/retnet/>). One of the challenges for the clinician is how to utilise  
72 the results of molecular genetic testing to improve clinical care and our understanding of  
73 disease mechanisms. Detailed knowledge of genotype/phenotype correlations allows targeted  
74 and therefore cost-effective molecular testing, clearly advantageous in a disorder with so  
75 many causative genes, and will help determine which disorders should be prioritised for the  
76 development of novel therapies.

77

78 Retinitis Pigmentosa with preservation of the para-arteriolar RPE(PPRPE) was first described  
79 by Heckenlively et al in 1982.[2] In this disorder there is relative preservation of retinal  
80 pigment epithelium (RPE) adjacent to and under retinal arterioles. In 1994, van den Born et  
81 al[3] reported a clinical study of 22 patients from one large family with PPRPE. Subsequent  
82 molecular genetic testing identified the disease locus on 1q31-32.1[4] and den Hollander et  
83 al[5] subsequently identified *CRB1* as the causative gene.

84

85 The *CRB1* gene is analogous to the *Drosophila Melanogaster* Crumbs protein that is involved  
86 in the establishment of the polarity of epithelial cells.[6,7] The CRB1 protein is found at the  
87 apical region of all retinal epithelial cells, the rod and cone photoreceptor cells and Muller  
88 glial cells(MGCs) in the adult mouse retina.[8] It co-localises with the zonula-adherens(ZA)  
89 and is a major component of the outer limiting membrane. Mutations in *CRB1* have been

90 identified in a variety of human retinal disorders including Leber congenital amaurosis  
91 (LCA),[9-11] PPRPE,[10] and retinitis pigmentosa.[12,13]

92

93 The aim of this study was to identify mutations in *CRBI* in a large cohort of patients and to  
94 characterise the ocular phenotype.

95

96

97 METHODS

98 **Patient recruitment:** Patients with a diagnosis of LCA or early childhood onset retinal  
99 dystrophy(EORD), and families with autosomal recessive RP where there was parental  
100 consanguinity were recruited from two centres – Moorfields Eye Hospital and Great Ormond  
101 Street Hospital for Children, London, UK. Ethics committee approval was obtained and  
102 research procedures were carried out in accordance with institutional guidelines and the  
103 declaration of Helsinki. Informed consent was obtained for all subjects prior to their  
104 participation in the study.

105

106 **Clinical Definitions:** All subjects had a family history consistent with AR inheritance.  
107 Patients were diagnosed as having LCA if vision was poor or absent in the first 3 months  
108 after birth, and where there was no recordable electroretinogram. A diagnosis of early onset  
109 retinal dystrophy was made when symptoms and/or signs were present before the age of 5  
110 years and subsequently, on electroretinography, there was evidence of a severe cone-rod or  
111 rod-cone dystrophy. Children older than 5 years with later onset of symptoms and signs of a  
112 retinal dystrophy were termed ‘juvenile onset’ or were labelled synonymously as autosomal  
113 recessive retinitis pigmentosa(ARRP).

114

115 **Clinical Evaluation:** A full clinical history and examination was performed in all subjects. In  
116 older children and adults this included best corrected LogMAR visual acuity and colour  
117 vision testing using the Hardy-Rand-Rittler pseudoisochromatic plates(Richmond Products).  
118 In the low vision range, for the purposes of later analysis, LogMAR values of 2.0 were  
119 applied for counting fingers acuity; 3.0 for hand movements, 5.0 for light perception and 6.0  
120 for no light perception.[14] Visual field testing using Goldmann perimetry was performed in

121 those with stable fixation. Subsequent analysis of the visual field area was performed using  
122 planimetry within the V4e isopter.

123

124 Refraction data were ascertained either by obtaining an objective cycloplegic refraction using  
125 neutralising lenses, or using an autorefractor(Luneau L62-3D autorefractokeratometer -  
126 LUNEAU Chartres, France). Fundus photographs were obtained where possible using the  
127 Topcon retinal camera(TRC-50IX with IMAGEnet 2000 system software – TOPCON  
128 corporation, Japan). Optical Coherence Tomography scans were performed on a Stratus  
129 OCT™(Carl Zeiss Meditec, Inc. Dublin, CA); further OCT imaging was performed in some  
130 patients using the Topcon 3d OCT-1000 and the Spectralis spectral domain OCT(Heidelberg  
131 engineering, Germany). Fundus autofluorescence images were obtained using the Heidelberg  
132 Retinal Angiograph-II(Heidelberg engineering, Germany). Axial length and keratometry  
133 measurements were obtained using the IOL Master(Carl Zeiss Meditec, Dublin CA). All  
134 patients had electrophysiological testing performed either at our institutions or at the referring  
135 hospital: many recordings were historical and either preceded ISCEV Standard recordings or  
136 incorporated the ISCEV standards pertaining at the time. Where further electrophysiological  
137 testing was required to make or confirm a diagnosis, adults and older children had  
138 electroretinography conducted in accordance with current ISCEV standards using gold foil or  
139 DTL electrodes.[15,16] ERG testing for infants and young children in our institutions was  
140 performed using skin electrodes in accordance with previously published protocols.[17,18]

141

142

143

144

145



146 **Genotyping**

147 DNA was extracted from whole blood obtained from each affected individual and immediate  
148 family members using the Nucleon BACC-2 kit(GE Healthcare) performed as per  
149 manufacturer's instructions.

150

151 DNA samples from patients with LCA and EORD were sent to Asper Ophthalmics(Tartu,  
152 Estonia) for analysis using the LCA chip.[19] In those subjects in whom a mutation in *CRBI*  
153 was identified, DNA samples were then sequenced to confirm the chip findings and, where  
154 only one mutation was found, to identify a second change.

155

156 DNA from consanguineous families with autosomal recessive RP were analysed using the  
157 Affymetrix 100K gene chip. Those showing linkage to the 1q31-32 region were sequenced  
158 for *CRBI* mutations. DNA samples from patients with recessive RP who had a retinal  
159 phenotype characteristic of *CRBI* were also sequenced.

160

161 Oligonucleotide primer pairs (appendix 1) were created to cover the exon and splice site  
162 junctions (based on RefSeq accession number NM\_201253.1) and were designed using  
163 Primer 3([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) and ordered from  
164 Sigma-Genosys(Sigma–Aldrich, UK). The amplified products were sequenced with Big Dye  
165 terminator v1.1 cycle sequencing kits(Applied Biosystems, Cheshire, UK) according to  
166 manufacturer's instructions. The pathogenic impact of the *CRBI* sequence variants caused by  
167 non-synonymous changes of the protein sequence was evaluated through SIFT  
168 (<http://sift.jcvi.org>) and PolyPhen(<http://genetics.bwh.harvard.edu/pph/index.html>) sequence  
169 homology-based programmes.

170

171 RESULTS

172 DNA samples from 250 probands with LCA/EORD were analysed using the LCA chip. 21  
 173 probands were found to have mutations in *CRB1*; 7 had two mutations and 14 had one  
 174 mutation identified. All mutations were confirmed by direct sequencing and a second  
 175 mutation was identified in 11 patients. Two recessive RP families, who were studied with  
 176 Affymetrix 100k gene chips, showed linkage to the RP12 locus and were found to have  
 177 homozygous mutations in *CRB1*. A further 14 RP patients with a clinical phenotype  
 178 suggestive of *CRB1* related disease were also sequenced; 9 were found to have mutations in  
 179 *CRB1* and two mutations were identified in 6 of these patients.

<i>Patient No.</i>	<i>Diagnosis</i>	<i>Exon</i>	<i>Nucleotide 1</i>	<i>Protein 1</i>	<i>Exon</i>	<i>Nucleotide 2</i>	<i>Protein 2</i>
1	EORCD	9	2843G>A	C948Y			
2	LCA	2	614T>C	I205T			
3	EORD	9	3037C>T*	Q1013X*			
4	ARRP	7	2129A>T	E710V*			
5	ARRP + retinal telangiectasia	7	2290C>T	R764C			
6,7	ARRP/LCA	9	3103C>T*	H1035Y*			
8,9	EORCD	7	2401A>T	K801X	12	4094C>A*	A1365D*
10-15	EOCRD/EORCD/ ARRP + retinal telangiectasia	3	750T>G	C250W	3	750T>G	C250W
16	LCA	7	2548G>A	G850S	9	3307G>C	G1103R
17	LCA	7	2401A>T	K801X	9	2843G>A	C948Y
18	EORCD	7	2290C>T	R764C	7	2401A>T	K801X
19	LCA	9	3542dupG*	C1181WfsX12*	9	3542dupG*	C1181WfsX12*
20	EORCD	9	2843G>A	C948Y	7	2222T>C	M741T
21	LCA	9	2843G>A	C948Y	3	717_718InsG*	Q240fsX20*
22	LCA	7	2548G>A	G850S	12	4142C>T*	P1381L*
23	LCA	9	2843G>A	C948Y	9	3655T>G*	C1174G*
24	LCA	9	2843G>A	C948Y	6	1576C>T*	R526X*

25	LCA	9	2843G>A	C948Y	9	2843G>A	C948Y
26	EORCD	9	2843G>A	C948Y	9	2843G>A	C948Y
27,28	LCA	7	2676 delG*	K892NfsX95*	7	2676delG*	K892NfsX95*
29	LCA	7	2548G>A	G850S	9	3008T>C*	I1003T*
30	ARRP	9	2843G>A	C948Y	7	2129A>T*	E710V*
31	LCA	9	2842G>A	C948Y	6	2043G>A*	C681X*
32	ARRP	7	2129A>T	E710V*	7	2234C>T	T745M
33	ARRP	7	2401A>T	K801X	9	3035T>C*	L1012S*
34	ARRP + retinal telangiectasia	9	3074 G>A*	S1025A*	9	3074 G>A*	S1025A*
35,36	EORCD	7	2536G>A	G846R	7	2536G>A	G846R
37	EORCD	2	470G>C*	C157S*	7	2506C>A	P836T
38	EORCD + retinal telangiectasia	4	936T>G*	N312K*	9	2843G>A	C948Y
39	LCA	7	2401A>T	K801X	9	3320T>C	L1107P
40	ARRP + retinal telangiectasia	6	2025G>T*	W675C*	9	3520 T>G	C1174G
41	EORCD+ retinal telangiectasia	7	2234C>T	T745M	8	2688T>A	C896X

180 Table 1

181

182 Of the cohort of 306 patients, 41 patients from 32 families were identified as having  
183 mutations of *CRBI*(Table 1). Thirty different sequence variants were identified, two thirds of  
184 which were in exons 7 & 9. Seventeen novel disease associated sequence variants were  
185 found. None of these variants were found in 100 normal controls, and appropriate segregation  
186 of alleles in family members was confirmed where DNA was available. The most common  
187 mutation identified was the p.C948Y variant which was present in 13 alleles of 11 patients.  
188 Six probands had one mutation found and in 26 probands both alleles were identified. Of the  
189 latter group, 7 had homozygous mutations and 19 had compound heterozygous mutations.  
190 Mutations in the *CRBI* gene were identified in 7% of the LCA/EORD cohort.

191

192 **Clinical Findings**

193 Thirty-four patients from 26 families had two CRB1 mutations identified. They ranged in age  
194 from 2-48 years(mean 19 years; 22 male, 12 female). Twenty-one patients were Caucasian,  
195 11 were from the Indian subcontinent and 2 were African. The median visual acuity for the  
196 whole cohort was 1.6 LogMAR. The median and range of LogMAR visual acuity  
197 measurements for each decade, are given in Table 2.

198

<b>Age range (years)</b>	<b>0-10 (n=7)</b>	<b>11-20 (n=15)</b>	<b>21-30 (n=5)</b>	<b>31-40 (n=4)</b>	<b>41-50 (n=3)</b>
<b>Median LogMAR VA</b>	1	0.95	2	3.5	5.5
<b>LogMAR VA range</b>	0.21 - 5(LP)	0.25 - 5 (LP)	0.8-3(HM)	0.34 - 6 (NLP)	5 - 6 (LP- NLP)

199 Table 2

200

201 The median spherical equivalent refraction for all patient measured was +1.5 dioptries.  
202 Funduscopy revealed the majority of patients to have nummular pigmentation at the level of  
203 the retinal pigment epithelium that was more widespread in the older patients. Ten  
204 patients(29%) had macular atrophy and 6(18%) had PPRPE. Five(15%) subjects had  
205 peripheral retinal telangiectasia, which in three cases was associated with exudative retinal  
206 detachment. Two patients had seclusio pupillae and secondary glaucoma. Median foveal  
207 thickness on Stratus OCT was 326µm(range 212-854µm; s.d. 148µm).

208

209

210

Diagnosis	Median age at examination (years)	LogMAR acuity	Spherical equivalent refraction Dioptres	Visual field size (square degrees inside V4e isopter)	Axial length mm	Foveal thickness on Stratus OCT ( $\mu$ m)	PPRPE	Macular atrophy	'Coats-like' vasculopathy
<b>LCA</b> (n=15)	10	2 (CF)	+6	100	18.70	264	N=2	N=7	N=1
<b>RCD</b> (n=7)	17	2 (CF)	+2	2500	20.2	316	N=1	N=3	N=1
<b>CRD</b> (n=3)	16	0.6	0	-	20.6	235	N=1	N=0	N=1
<b>ARRP</b> (n=9)	20	0.8	+1	4000	20.7	345	N=2	N=0	N=2

211 Table 3

212

213 The LCA patients all had a non-detectable ERG. Visual acuity ranged from 0.9 LogMAR to  
214 NPL. Refraction ranged from +4.5 to +9.5 dioptres (spherical equivalent), and correlated with  
215 short axial length, which ranged from 17.95mm to 23.06mm(Table 3). Colour vision was  
216 absent in all but one patient who was able to discern the highest contrast HRR plates.  
217 Reliable OCT measurements could only be made in 6 patients because of nystagmus; foveal  
218 thickness ranged from 212 $\mu$ m to 854 $\mu$ m with intraretinal cystic spaces seen in two patients.  
219 Funduscopy showed white dots at the level of the RPE in the younger patients and deep  
220 nummular pigment in older subjects(Figure 1D). Macular atrophy was present in 7 of the  
221 15(46%) patients with LCA(Figure 1F). Analysis of mutations in this group of patients  
222 showed that 23/24 alleles were either protein truncating(9/24) - both nonsense and frame-  
223 shifting - or predicted by both SIFT and PolyPhen analysis to severely affect protein function  
224 (14/24).

225

226 The early onset rod-cone dystrophy patients were between 5 and 39 years of age when  
227 examined. Nine patients had a historical diagnosis of ARRP, but on further questioning had  
228 symptoms that were childhood or juvenile in onset. The median onset of symptoms was at 2.5  
229 years(range 6 months to 5 years). The median age at diagnosis was 6 years. Visual acuities  
230 ranged from 0.16 LogMAR to PL. The spherical equivalent refractive error ranged from  
231 -0.5D to +6.5D with the majority having low hyperopia. Axial lengths ranged from 19.03mm  
232 to 23.35mm. The foveal thickness measurements, on Stratus OCT, ranged from 217-396µm.  
233 Intraretinal cysts were common. (Figure2B,C,D). The visual field was relatively well  
234 preserved in several patients and field area ranged from 102-10000 square degrees. Fundus  
235 examination revealed widespread pigment clumping at the level of the RPE in the majority of  
236 cases. One patient exhibited PPRPE (Figure1C).

237

238 The 3 patients with cone-rod dystrophy were aged 13, 17 and 19 years at examination. Poor  
239 central vision was the first symptom with later development of night blindness and visual  
240 field loss. One patient had peripheral retinal telangiectasia and exudative retinal detachment  
241 (Figure1A; Figure2E). The ERG findings suggested generalised photoreceptor dysfunction  
242 with evidence of more severe macular involvement (Figure3).

243

244 Examination of the mutations in these two groups of early onset patients showed 16/18 alleles  
245 would be predicted to affect protein function severely, similar to the pattern seen in the LCA  
246 group. Three of 18 alleles contained nonsense mutations; 13/18 alleles contained mis-sense  
247 mutations predicted to have a severe effect on protein function.

248

249 The patients with juvenile onset rod-cone dystrophy had a less severe phenotype and later  
250 onset of disease. The median age at diagnosis was 10 years. Visual acuities ranged from 0.28

251 LogMAR to NPL, with the median acuity 0.8 LogMAR. Low hypermetropia was noted in all  
252 but one patient. Visual field planimetry ranged in size from 620-11160 square degrees.  
253 Foveal thickness, measured by OCT, ranged from 322-544  $\mu\text{m}$  with several patients  
254 exhibiting intra-retinal cysts. The retinal appearances were more heterogeneous and ranged  
255 from a grey discolouration of the RPE around the arcades with PPRPE to extensive retinal  
256 pigmentation. Retinal telangiectasia was seen in 2 patients who subsequently developed  
257 retinal detachment, uveitis with seclusio pupillae, and angle closure glaucoma. In this group  
258 of patients there was a lower prevalence of protein truncating mutations and a higher  
259 prevalence of missense mutations compared to those subjects with LCA.

260

## 261 DISCUSSION

262

263 The present study investigated the incidence of *CRBI* mutations in a large cohort of patients  
264 with recessive retinal dystrophies reports 17 novel mutations. An APEX microarray was used  
265 as an initial screening strategy and *CRBI* mutations were identified in 7% of patients with  
266 LCA and EORD. This is likely to be an underestimate, as sequencing of *CRBI* was not  
267 performed in all cases. When cases with a suggestive clinical phenotype were also included  
268 the detection rate in this cohort increased to 12%, indicating that *CRBI* mutations are a  
269 common cause of LCA and childhood onset rod cone dystrophies.

270

271 Mutations in *CRBI* account for 10-13% of patients with LCA,[9-11] but *CRBI* mutations  
272 have also been identified in patients with autosomal recessive retinitis pigmentosa  
273 (RP12)[5,20] where there is often para-arteriolar sparing of the RPE. A *CRBI* mutation has  
274 also been reported in a single family with autosomal dominant pigmented paravenous  
275 chorioretinal atrophy (PPCRA) that exhibited variable expressivity.[21] Peripheral retinal

276 telangiectasia and associated exudation is more commonly associated with *CRBI* mutations  
277 than other forms of RP.[3,13] We observed telangiectasis, exudation and subsequent  
278 development of retinal detachment in 15% of our cohort. It seems plausible that this ‘Coats-  
279 like’ exudative vasculopathy is secondary to a breakdown in the normal blood retinal barrier  
280 mediated by loss of normal zonula occludens function and therefore immune exposure to  
281 retinal antigen.[22] The characteristic appearances of exudation and retinal detachment,  
282 yellow lipid deposition, with subsequent uveitis and neovascular glaucoma would seem to be  
283 a final common pathway of abnormal retinal development whether the cause is secondary to  
284 mutations in *CRBI*, *Norrin*, or associated with FEVR and Coats disease.

285

286 We identified *CRBI* mutations in patients with LCA, early onset rod-cone dystrophy, juvenile  
287 onset RP, and cone rod dystrophy. The severity of the retinal disease generally correlated  
288 with the age of onset of symptoms. The disease appears clinically progressive though  
289 longitudinal data was not collected; however, all patients examined who were in their fourth  
290 decade had visual acuities of CF or worse. There is a readily recognisable phenotype that can  
291 help guide molecular genetic testing. In infants there are typically widespread sub-retinal  
292 white dots and macular atrophy is common. The characteristic deep nummular pigmentation  
293 usually develops in later childhood and the pigmentation becomes more extensive with age.  
294 PPRPE, present in 18% of our patients, is strongly suggestive of *CRBI* related disease,  
295 particularly when accompanied by peripheral telangiectasia. A variety of macular changes  
296 were found: macular atrophy and loss of central macular volume on OCT was noted  
297 particularly in patients with LCA and early onset rod-cone dystrophy. No clear relationship  
298 with genotype was established though it was noted that 6 of the 13 patients in the cohort that  
299 had either heterozygous or homozygous frame-shifting/termination mutations had macular  
300 atrophy noted on funduscopy.



301

302 OCT imaging is also extremely helpful in identifying *CRB1* related disease. Jacobson et  
303 al[23] first described increased retinal thickness and loss of the normal retinal laminae in  
304 patients with *CRB1* mutations. All of the patients in the current study who had OCT showed  
305 significantly increased retinal thickness. Newer spectral domain OCT imaging permits better  
306 visualisation of the laminar arrangement of the inner retina. In one family, with three affected  
307 individuals, spectral domain OCT showed well delineated retinal layers with preservation of  
308 the outer limiting membrane (OLM) in the younger patients but coarse lamination and loss of  
309 the OLM in the oldest subject (Figure2B,C,D). This suggests that the coarse lamination seen  
310 on OCT may not represent a developmental abnormality, but that loss of the normal  
311 architecture develops with time. The *CRB1* protein is known to play a critical role in retinal  
312 development[6,8,24] and patients with *CRB1* mutations have abnormal retinal structure as a  
313 consequence. Longitudinal studies are required to determine whether there is progressive loss  
314 of the OLM, and what role this may have in determining the increased retinal volume seen on  
315 OCT.

316

317 The preliminary results of gene replacement therapy for LCA associated with mutations in  
318 *RPE65* have been promising[27-29] and this has led to optimism that similar treatment  
319 strategies may be helpful in other forms of LCA and childhood retinal dystrophy. The retinal  
320 disease associated with *CRB1* mutations is generally more severe but there are some patients  
321 in whom there is a window of opportunity for therapy in early childhood.

322

323

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EXON/ AMPLIME	5' FORWARD	3' REVERSE	ANNEALIN G TEMP
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R			
1	CCTCCCGTGTAAGTGATGCT	AACTGACTGTTCACATTGACTG G	60.6
2.1	GCAGCACAAAGGTCACAAAG	GCAGGGATCTGAAGCACATT	65.8
2.2	GGAGGTATTTGCCATCAGGA	TGACAGCAGGCAACCACTTA	62.5
3	CTCTGCCTTGTGCAACTTCC	TAAGCCGAGAACGTGAGAGC	64.3
4	CCATGGGTCTTGGGTTGATA	GCAGTAAATATGTGTGAGCAC TGA	64.3
5	CCTCCTTTTAGGCAAATGCTC	TACTTTTCATGCCACAAGG	58.7
6.1	GCTATTCATGCACTTCTGCAA	GCTCCAGCTTCACAAACACA	60.6
6.2	ACTTTTCATTTGAGGGCGATG	ACAAGGTTGGCTTTCACACC	64.3
6.3	GATGGAGAGTGGCATTTCGT	TTTCATAGCAGGCAGAAGCA	64.3
7.1	TTCGTCTTCCATCCCTTCTG	GTCAGGTAGGCCACCAATGT	64.3
7.2	GCAATGCTGACTCCAAACTCT	GGCCTCATTCTATGGCTCA	58.7
8	ATGTGGTTTCACCGTCAACA	AGGCAAGAGGCCAGTCAGTA	64.3
9.1	ATGTATCAAATAGTCAATATGCAA TGT	TCAAAAAGTTGAGGCTTCCAG	65.8
9.2	TGGAAGTGGACAACGAAACA	TTCCAAAACAGAGGCAAGAA	58.7
9.3	TTGCATGGAGGAAACTGTGA	CAGTGTCACCCTGTTTCAGCA	64.3
10	CCTCCAGCAGGAGCTTTTTA	AAAATGTTCTCCCCTTTGACC	60.6
11	AGGGACCTGGGTTTCTGCT	GGGAAGAAATTGCAGTGGTC	64.3
12.1	GCTTGCTCTGGTTGGTCTC	GCGGAACCACTGTGAAAGTT	64.3
12.2	TGACAATGTTAATCTGCAACTGG	AAGCAGGTTCTTCAAATGTTCC	64.3

422 Appendix 1

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