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1 **A novel role for CRIM1 in the corneal response to UV and pterygium development**

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24

25 **ABSTRACT**

26 Pterygium is a pathological proliferative condition of the ocular surface, characterized by formation  
27 of a highly vascularized, fibrous tissue arising from the limbus that invades the central cornea leading  
28 to visual disturbance and, if untreated, blindness. Whilst chronic ultraviolet (UV) light exposure plays  
29 a major role in its pathogenesis, higher susceptibility to pterygium is observed in some families,  
30 suggesting a genetic component.

31 In this study, a Northern Irish family affected by pterygium but reporting little direct exposure to UV  
32 was identified carrying a missense variant in *CRIMI* NM\_016441.2: c.1235 A>C (H412P) through  
33 whole-exome sequencing and subsequent analysis. *CRIMI* is expressed in the developing eye, adult  
34 cornea and conjunctiva, having a role in cell differentiation and migration but also in angiogenesis,  
35 all processes involved in pterygium formation. We demonstrate elevated *CRIMI* expression in  
36 pterygium tissue from additional individual Northern Irish patients compared to unaffected  
37 conjunctival controls.

38 UV irradiation of HCE-S cells resulted in an increase in ERK phosphorylation and *CRIMI* expression,  
39 the latter further elevated by the addition of the MEK1/2 inhibitor, U0126. Conversely, siRNA  
40 knockdown of *CRIMI* led to decreased UV-induced ERK phosphorylation and increased *BCL2*  
41 expression.

42 Transient expression of the mutant H412P *CRIMI* in corneal epithelial HCE-S cells showed that,  
43 unlike wild-type *CRIMI*, it was unable to reduce the cell proliferation, increased ERK  
44 phosphorylation and apoptosis induced through a decrease of *BCL2* expression levels.

45 We propose here a series of intracellular events where *CRIMI* regulation of the ERK pathway  
46 prevents UV-induced cell proliferation and may play an important role in the in the pathogenesis of  
47 pterygium.

48

49 **Keywords:** CRIM1; pterygium; UV; proliferation; ERK; apoptosis; variant

50

51 **Abbreviations:** **BCL2**, B-cell lymphoma 2; **BMP**, Bone Morphogenetic Proteins; **CRIM1**, Cysteine Rich Motoneuron protein1; **EMT**, Epithelial

52 mesenchymal transition; **ERK (I)**, Extracellular signal–regulated kinases (Inhibitor); **HCE-S**, Human Corneal Epithelial cells; **IC**, Impression

53 Cytology; **IGV**, Integrative Genomic Viewer; **MAF**, Minor allele frequency; **MAPK**, Mitogen-activated protein kinases; **MTT**, 3-(4,5-

54 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NGS**, Next Generation sequencing; **NI**, Northern Irish; **PolyPhen**, Polymorphism

55 Phenotyping; **SIFT**, Sorting Intolerant From Tolerant; **TiGER**, Tissue-specific Gene Expression and Regulation; **TGF- $\beta$  (I)**, Transforming Growth

56 Factor-  $\beta$  (Induced); **TUNEL**, Terminal deoxynucleotidyl transferase dUTP nick end labelling; **VEGFA**, Vascular Endothelial Growth FactorA;

57 **VW(F)-C**, Von Willebrand (Factor) C; **WES**, Whole Exome Sequencing.

58

## 1. INTRODUCTION

Pterygium (OMIM 178000) is a triangular shaped proliferative fibrovascular growth arising from the corneal-scleral limbus that invades the cornea centripetally<sup>1,2</sup>. The corneal invasion, through an active process of cell proliferation, matrix remodelling, angiogenesis and inflammation<sup>1,3</sup>, results in astigmatism, irritation, tearing, and, if the visual axis is impinged upon, blindness in the most severe cases<sup>4</sup>. The only effective treatment for pterygium is surgical excision which, together with various adjuvant therapies, still presents a 12% recurrence rate<sup>5</sup>. A small conjunctival lesion, pinguecula, is considered to be related to pterygium with a similar aetiology<sup>6</sup>, limbal localisation<sup>7</sup> and histology<sup>8</sup>.

The main trigger for pterygium has long been attributed to UV radiation<sup>9-11</sup>, with epidemiologic studies showing an average prevalence of 22% (as high as 40% in some Chinese populations<sup>12</sup> or 45% in provincial Indonesia<sup>13</sup>) in the “pterygium belt”, an equatorial zone between latitudes 40°N and 40°S, compared to only 2% outside this area<sup>4</sup>. The human cornea represents a shield to protect the anterior eye from UV light and its anisotropic properties ensure that UV transmittance is reduced with a lower wavelength: while UVB (290-320nm) is completely absorbed by corneal epithelium, UVA (320-400nm) is absorbed only by 20% by the epithelial layer with the rest reaching the underlying stroma<sup>14</sup>. Focussing of incident light upon the nasal limbus has been proposed as the mechanism for the more frequent occurrence of pterygia at this location<sup>15</sup>.

In pterygium, while UVB mediates oxidative DNA damage<sup>16,17</sup> and induction of cytokines and growth factors<sup>18</sup>, both UVB<sup>19</sup> and UVA<sup>20</sup> are responsible for activation of ERK intracellular pathway.

Irritation and chronic inflammation caused by sand, dust and wind<sup>21</sup>, as well as viral infections<sup>22,23</sup>, have been suggested as additional environmental triggers for pterygium.

A genetic contribution to the incidence of pterygium was suggested in an Australian study<sup>24</sup>, which revealed that 38% of patients admitted to hospital for excision of pterygia reported relatives

84 with the disease, and in studies where different ethnic groups living at the same geographical  
85 location display differing prevalence of pterygium<sup>12,25</sup>.

86 High incidence of pterygium has been reported in a number of multigenerational families<sup>26,27</sup> and  
87 in monozygotic twins<sup>28,29</sup>, where the most commonly reported mode of inheritance is autosomal  
88 dominant with reduced penetrance<sup>4,30,31</sup>.

89 The lack of large families presenting with multiple affected individuals, compounded by the late-  
90 onset of the disease, together with incomplete penetrance, has hampered identification of  
91 causative genes for pterygium.

92 Candidate gene association studies, which derive from hypotheses in which the cause of  
93 pterygium is already assumed, have identified an increased predisposition to pterygium in  
94 individuals carrying germline mutations in genes related to: oxidative stress<sup>16,32</sup>, carcinogenesis<sup>33</sup>  
95 or angiogenesis<sup>34</sup>; even though these analyses have been found subject to false-positive  
96 associations<sup>35</sup>. Next-generation sequencing techniques, such as Whole Exome Sequencing (WES)  
97 and Whole Genome Sequencing (WGS), provide a largely hypothesis-free approach to identifying  
98 causative genes in smaller families with fewer affected members<sup>36</sup>.

99 In this study, using a WES approach, combined with bioinformatic and functional analysis, we  
100 identified a missense variant, p.His412Pro, in the *CRIMI* (cysteine rich motor neuron protein 1)  
101 gene, found in pterygium affected members of large multigenerational Northern Irish family with  
102 a documented small exposure to sunlight. *CRIMI* response to UV exposure revealed, for the first  
103 time, a central role within an intracellular mechanism involving ERK phosphorylation and  
104 ultimately leading to cellular proliferation or apoptosis. Introduction of the H412P variant in  
105 *CRIMI* resulted in an impairment of the whole pathway, demonstrating its possible involvement  
106 in the NI family's pterygium pathogenesis.

107  
108

109 **2. MATERIALS AND METHODS**

110 **2.1 Patient clinical examination**

111 Clinical examinations were performed at Cathedral Eye Clinic, Belfast, UK (pterygium family  
112 members, pterygium and control individual samples from Northern Ireland).

113 A total of 24 patients from three consecutive generations of a Caucasian Northern Irish family  
114 affected by pterygium were investigated: three with pterygium, two with pinguecula and one  
115 unaffected family member participated to the WES study. Additional Northern Irish and Bolivian  
116 individuals (pterygium affected and unaffected controls) were recruited for sequence analysis of  
117 *CRIMI* and *CRIMI* expression analysis.

118 Following informed consent, collection of blood, tissues and a completed questionnaire was  
119 obtained from each participating individual under ethical approval from ORECNI Northern  
120 Ireland, UK (09/NIR01/14) and Comité de Bioética de la Facultad de Medicina, Santa Cruz,  
121 Bolivia.

122 **2.2 Whole Exome Sequencing and Ingenuity Variant Analysis**

123 WES was performed at the Wellcome Trust Centre for Human Genetics, University of Oxford.  
124 Briefly, genomic DNA was extracted from blood leukocytes using the QIAamp DNA Blood mini  
125 kit (QIAGEN, Manchester, UK), quantified by the high sensitivity Qubit system (Thermo Fisher  
126 Scientific, Loughborough, UK) and integrity of the DNA confirmed by electrophoresis on a 1%  
127 agarose gel.

128 The SureSelect Human All Exon v2 kit was used for Whole Exome capture according to the  
129 manufacturer's instructions (Agilent Technologies UK, Wokingham, Berkshire, UK). The  
130 SureSelect n.2100 Bioanalyser (Agilent Technologies) allowed an assessment of the quality of  
131 the exome enriched library obtained.

132 Parallel sequencing was then performed by Illumina GAIIx using 150bp-paired-end reads.  
133 Generated reads were aligned to the Human 37 reference genome with a short read mapper  
134 (Stampy) generating data in BAM format<sup>37</sup>.  
135 Coverage of the target region was verified to be in excess of 70% (greater than 10 reads). Platypus,  
136 an in-house variant caller able to detect Single Nucleotide Variants (SNVs) and short (<50bp)  
137 insertion/deletions (INDEL), was used to detect variant sites and alleles. Once the false positive  
138 rate was reduced, the resulting variants were stored as Variant Call Format (VCF) files. Ingenuity  
139 Variant analysis (Qiagen) was used to filter and select a smaller number of candidate genes.  
140 Aligned WES reads were viewed with the IGV platform. Clustal X2.1 was used to align CRIM1  
141 sequences and results were visualized with EsPript 3.0 (<http://espript.ibcp.fr>)<sup>38</sup>.

### 142 **2.3 Sanger Sequencing**

143 Sanger sequencing was performed on genomic DNA extracted from the blood leukocytes using  
144 QIAamp DNA Blood mini kit.  
145 Presence of H412P in *CRIM1* was verified using the following primers: CRIM1\_F:  
146 CTTCTTTTGCATGCACCCCC and CRIM1\_R: TCACATGTGCAACCTTTCCTC while  
147 *CRIM1* VWFs were sequenced using genomic primers designed using Primer3<sup>39</sup> (Supplementary  
148 Table2). The PCR products were verified on a 1% agarose gel and Sanger sequenced at the  
149 Department of Zoology, University of Oxford.

### 150 **2.4 Tissues and Impression cytology samples**

151 Tissues samples (pterygium and normal cornea) were collected during surgeries while impression  
152 cytology samples were obtained using 4 x 4 mm strips of sterile LCR biopore membrane filter  
153 (pore size, 0.45 um; Millipore, Watford, UK) post pterygium surgery as previously described<sup>40</sup>  
154 and stored in RNAlater (Qiagen). Prior to IHC staining tissues were fixed in 95% ethanol for 20  
155 minutes at room temperature. The RNA yield obtained from the impression cytology samples was  
156 never lower than 8ng/ul and never higher than 15ng/ul. The ratio of absorbance at 260 and 280

157 nm (A260/280) of the RNA samples was between 1.8 and 2.2. Samples for which the RNA yield  
158 was too low or the quality fell outside the limits were excluded.

159

## 160 **2.5 Quantitative real-time PCR**

161 RNA extracted using the RNeasy Plus Mini Kit (Qiagen, Manchester, UK) was quantified with  
162 the Nanodrop 1000 (Thermo Fisher Scientific) and 1 µg of total RNA was reverse transcribed  
163 into cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Paisley,  
164 UK). cDNA was used for qRT-PCR assays, performed using a Lightcycler 480 II (Roche, West  
165 Sussex UK).

166 Real Time Ready Assays for *CRIMI* (assay id. 112278), *VEGFA* (assay id. 140396), *SRCAP*  
167 (assay id. 126413), *TGF-β1* (assay id. 104720), Glyceraldehyde 3-phosphate dehydrogenase  
168 (*GAPDH*) (assay id. 141139) and hypoxanthine phosphoribosyltransferase (*HPRT*) (assay id.  
169 102079) were purchased from Roche (Burgess Hill, West Sussex, UK).

170 SYBR green (Fermentas, Cambridge, UK) technology qRT-PCR was performed using *BCL2*  
171 primers (For AGCATGGGAGCCACGACCCT, Rev GGCCAAGGCCACACAGCCAA) and  
172 *HPRT* primers (For AGCTTGCGACCTTGACCAT, Rev GACCACTCAACAGGGGACAT), a  
173 kind gift from H. Nesbitt<sup>41</sup>.

174 Data were normalised using *HPRT* and *GAPDH* as housekeeping controls for  $\Delta$ Ct and  $\Delta\Delta$ Ct  
175 calculations<sup>42</sup>. *HPRT* and *GAPDH* were chosen as they have been shown to be the most stable  
176 corneal housekeeping genes<sup>43</sup>. For each condition all complementary cDNA samples were run in  
177 duplicate in two independent experiments.

## 178 **2.6 HCE-S culture**

179 Human Corneal Epithelial cells (HCE-S), a spontaneously generated corneal cell line<sup>44</sup> (a kind  
180 gift from Prof. Julie Daniels), were cultured (37°C, 5% CO<sub>2</sub>) in Dulbecco's modified Eagle's

181 medium (DMEM) containing 4 g/L glucose (Thermo Fisher Scientific, UK), and supplemented  
182 with 10% fetal bovine serum (Thermo Fisher Scientific, UK).

### 183 **2.7 TUNEL assay**

184 The terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was  
185 performed on HCE-S cells which had been reverse transfected with Lipofectamine 2000 using a  
186 mock transfection with pGL4.17 [luc2/Neo] plasmid (Promega Madison, WI USA), *CRIMI* wild  
187 type or *CRIMI* H412P plasmids. After 72 hours cells were fixed with 4% paraformaldehyde  
188 (PFA) and stained using the In Situ Cell Death Detection kit (Fluorescein; Roche, Burgess Hill,  
189 Surrey, UK) following the manufacturer's instructions. Images were obtained using a fluorescent  
190 AxioScope A1 microscope equipped with an AxioCam MRc camera (Carl Zeiss, Germany); 10x  
191 objective. Twelve images for each condition (n=3) were quantified using ImageJ software (US  
192 National Institutes of Health) and then normalised to total DAPI cells in each field.

### 193 **2.8 Immunohistochemistry (IHC)**

194 Pterygium and conjunctival tissues were formalin fixed and paraffin embedded. Sections of 7µm  
195 were permeabilised with 0.5% Triton X-100 before staining, treated with Proteinase K solution  
196 (Fisher Bioreagents, BP1700-50, 10ug/ml in PBS) and blocked with 5% goat serum (Sigma). A  
197 rabbit polyclonal CRIM1 antibody (Abcam- ab189203) was incubated at 1:100, with a rabbit IgG  
198 (Abcam) used as an isotype control. Secondary antibody fluorescein isothiocyanate (FITC)-  
199 conjugated goat anti-rabbit IgG (Santa Cruz, USA) was used at a 1:100 dilution. Each section was  
200 mounted with fluorescent mounting medium (DAKO) and imaged using a 20× N Archoplan lens  
201 on an AxioScope.A1 microscope equipped with an AxioCam MRc camera (Carl Zeiss, Germany).

### 202 **2.9 Site Directed Mutagenesis**

203 *Human CRIMI* cloned into a pcDNA3.1 plasmid was a kind gift from Dr. L Wilkinson, Institute  
204 for Molecular Bioscience, University of Queensland, Brisbane, Australia<sup>45</sup>. Site directed  
205 mutagenesis was performed to obtain the H412P *CRIMI* clone, using the Quick Change II kit

206 (Agilent Technologies), following the manufacturer's instructions. The entire *CRIMI* sequence  
207 was checked for integrity by Sanger Sequencing (Department of Zoology, University of Oxford),  
208 primers used are listed in Supplementary Table1.

## 209 **2.10 MTT assay**

210 Reverse transfection was performed in 12 well plates using HCE-S cells with either  
211 pCas9D10A\_GFP (Addgene/Zhang lab), *CRIMI* wild-type or H412P plasmids with  
212 Lipofectamine 2000, according to the manufacturer's instructions (Falcon #353043, BD Corning  
213 Life Sciences, MA, USA). The following day cells were transferred in 96 well plates and 3-(4,5-  
214 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the medium.  
215 Following 2 hours of incubation, the cells were resuspended in DMSO (Dimethyl sulfoxide,  
216 Sigma), absorbance was measured at 570 nm using the FLUOstar Omega (BMG Labtech,  
217 Aylesbury, UK) and quantified as relative percentages compared to control conditions.  
218 Absorbance measurements for the MTT were taken at 24, 48, 72 and 96 hours post transfection.

## 219 **2.11 Western Blotting**

220 HCE-S cells were cultured as described above. Low cell seeding densities were used to avoid  
221 cells becoming too confluent as this causes a decrease in ERK phosphorylation independent of  
222 the effect of CRIM1<sup>46,47</sup>.

223 Proteins were extracted using Complete Lysis-M (Roche Diagnostics) and proteinase inhibitor  
224 (Sigma-Aldrich P8340), quantification of total protein was performed using the Bradford assay  
225 (BioRad), 25µg of the extracted proteins was then resolved on a 4-12% NuPAGE® Bis-Tris  
226 Precast Gels (Thermo Fisher Scientific UK) using NuPAGE® MOPS SDS Running Buffer and  
227 transferred onto Amersham TM Hybond ECL membrane (GE Healthcare Life Sciences).

228 Phospho ERK (#9101) and ERK (#9102) primary antibodies (Cell Signalling) were used at  
229 dilutions of 1:100 and 1:500 respectively with an overnight incubation at 4°C. A secondary swine  
230 anti-rabbit antibody (DakoCytomation, Ely, UK) was used at a 1:2000 dilution for 1 hour at room

231 temperature. Protein binding was detected by standard chemiluminescence: SuperSignal™ West  
232 Pico Chemiluminescent Substrate (Thermo Fisher Scientific UK) and imaged using the G:BOX  
233 transilluminator (Syngene). Quantification was performed using GeneTools image analysis  
234 software: average peak values of phosphoERK were normalised against the average ERK values.  
235 All the results obtained were then normalised to the transfection control.

## 236 **2.12 UV treatment and MEK1/2 inhibitor**

237 HCE-S cells were seeded in a 24-well plate at  $1 \times 10^5$  cells per well in growth medium and left to  
238 adhere overnight at 37°C and 5% CO<sub>2</sub>. The following day they were treated using a UVA cross-  
239 linker (IROC Innocross AG, Ramsen, Switzerland) delivering a dose of 5.4 J/cm<sup>2</sup> as previously  
240 described<sup>48</sup> or UVB at a final dose of 0.5 J/cm<sup>2</sup> through Arcadia D3 6% lamp (Arcadia, UK) with  
241 an aluminium reflector at a distance of 15 cm from the cells for 34 minutes.

242 The same doses of UVA and UVB irradiation were used in experiments in which the inhibitor of  
243 ERK phosphorylation (MEK1/2 inhibitor, U0126) was added to culture media an hour prior to  
244 the UV treatment as previously described<sup>20</sup>, at a concentration of 10µM.

245 After irradiation, HCE-S cells were incubated in culture medium at 37°C with 5% CO<sub>2</sub> and  
246 harvested at 1, 6, 12, 24 and 48 hours.

## 247 **2.13 siRNA transfection**

248 Four different siRNAs targeting the *CRIM1* sequence (Set of 4 Upgrade: ON-TARGETplus  
249 CRIM1 siRNA, LU-008492-00-0002, 2nmol, Dharmacon) were reverse transfected in HCE-S  
250 cells using Lipofectamine RNAiMAX (Fisher Thermo Scientific), following the manufacturer's  
251 instructions. The four siRNAs were transfected singularly or as a pool at a final concentration of  
252 10nM and normalised to the results from a non-specific siRNA control (NSC4)<sup>49</sup>. A range of  
253 different concentrations (0.2-0.5-1-10nM) of the siRNA pool reverse transfected in HCE-S cells  
254 was subsequently tested.

255

## 256 **2.14 Statistical Analysis**

257 All error bars represent the standard error of the mean (SEM) calculated between sample  
258 replicates of the same biological group. qRT-PCR, MTT and TUNEL assays significance was  
259 estimated using a Student's t-test calculation or the Mann-Whitney U test in GraphPad Prism 5  
260 software with data illustrated using Box plots (qRT-PCR in Fig.3). p value  $\leq 0.05$  was deemed to  
261 be significant (\*p value  $\leq 0.05$ , \*\*p value  $\leq 0.01$  and \*\*\*p value  $\leq 0.001$ ).

262

## 263 **3. RESULTS**

### 264 **3.1 WES analysis in a Northern Irish family affected by pterygium and pinguecula**

265 A multigenerational Northern Irish family presenting with pterygium and pinguecula, but not  
266 other eye abnormalities, was examined (Fig.1A). Pterygium affected both males and females and  
267 was diagnosed at an average age of 48 years. No history of unusual sun exposure was recorded  
268 for any family member, suggesting a familial predisposition for development of  
269 pterygium/pinguecula (Table 1).

270 Six members of this family underwent WES: three were affected by pterygium (II.2, II.4 and  
271 II.14, 72, 70 and 65 years old respectively), two by pinguecula (III.5 and III.6, 48 and 46 years  
272 old respectively), shown in Fig.2B, and one was unaffected (II.9, 58 years old). The other two  
273 younger unaffected members of the family (III.2 and III.3, age 49 and 34) did not participate in  
274 the WES but were subsequently analysed by Sanger Sequencing.

275 WES resulted in the identification of 451,153 variants in 18,858 different genes which were  
276 filtered in a stepwise manner by Variant analysis software (Ingenuity®) (Fig.1C). Selecting only  
277 those variants with a call quality  $\geq 50$  in any case and  $\geq 20$  in the control and a read depth  $\geq 10$ ;  
278 30,000 variants were obtained. When variants with a minor allele frequency (MAF) greater than  
279 0.5% (pterygium prevalence in Europe is 2%) in 1000 Genomes Project, ESP EA exomes and

280 Complete Genomics genome were excluded, the number of variants was reduced to 25,000.  
281 Analysis using either of the two algorithms Polyphen and SIFT, predicted as deleterious 11,000  
282 variants, while 40 variants were finally selected as displaying an autosomal dominant segregation  
283 pattern within the family (Table 2).  
284 Genes carrying mutations previously associated with pterygium such as *Ku70*, *GSTM1*, *ACE*  
285 *hOGGI*<sup>16,17,33,34</sup> were not observed in the selected list of variants.  
286 Each single variant obtained by this analysis was then manually reviewed for determination of  
287 protein structure/function in Polyphen and SIFT, conservation of the amino acid among species  
288 running BLAST or eye expression interrogating Tiger Expression database. Literature was  
289 reviewed for any possible known correlation between gene mutations and pterygium or other  
290 diseases, either of the eye or otherwise. Moreover, adequate sequencing coverage and co-  
291 segregation of the variant within the family were verified through Integrative Genomic Viewer  
292 (IGV) (Table 2).  
293 Based on the gene expression profile and on the known role in the eye, we selected *CRIM1* as our  
294 most plausible candidate gene. Recent studies have elucidated the importance of CRIM1  
295 expression in corneal and conjunctival development<sup>50-52</sup> and the same variant, H412P, was  
296 identified from another WES study on patients affected by Coloboma eye developmental  
297 disorder<sup>53</sup>. Moreover, CRIM1 revealed a role in cell proliferation<sup>54-56</sup>, adhesion and migration<sup>57-</sup>  
298 <sup>59</sup>, angiogenesis through VEGFA<sup>60-62</sup> and UV-related diseases<sup>63,64</sup>, the main processes involved  
299 in pterygium development<sup>4</sup>.

300

### 301 ***3.2 CRIM1 structure and sequence analysis in individuals from Northern Ireland and Bolivia.***

302 Presence of the variant in each affected family member, and absence from the unaffected sibling  
303 (II.9), was confirmed by direct Sanger sequencing (Fig.2A, example) of PCR products spanning  
304 the variant in VWFC-2.

305 Sequence alignment of *CRIM1* orthologues revealed residue H412 being conserved throughout  
306 human (*Homo sapiens*), cattle (*Bos Taurus*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), frog  
307 (*Xenopous laevis*), zebrafish (*Danio rerio*) (Fig.2B).

308 CRIM1 is a 1002 amino acid transmembrane protein (once the 34 amino acid signal peptide has  
309 been cleaved) and consists of a large N-terminal extracellular portion composed of 11 domains  
310 (six Von Willebrand factor C, four antistasin-like and one Insulin-like Growth Factor-binding  
311 Protein (IGFBP)), a 21 amino acids transmembrane domain and a small 76 amino acid C-terminal  
312 cytoplasmic domain<sup>65</sup> (Fig.2C).

313 Although H412, located in the second VWFC domain, is not conserved in the other five VWFC  
314 of *CRIM1* (Fig.2D), it is conserved in the VWFC-2 domain of human chordin and in the VWFC-  
315 2 and 3 domains of human neuralin<sup>66</sup>, two related bone morphogenic protein (BMP) antagonists,  
316 like CRIM1, all of which have a proven role during embryogenesis<sup>45</sup> (Fig.2E).

317 Sequence analysis of VWFC-2 in two additional younger family members (III.2 and III.3, age 49  
318 and 34) revealed them to possess the same H412P variant; despite these individuals not having  
319 developed any signs of the disease. This is not incompatible with the mutation predisposing to the  
320 pathology, since a pattern of reduced penetrance has been widely described in familial  
321 pterygium<sup>1,4,26</sup>.

322 All the CRIM1 VWFC domains are important for interaction with BMPs 4 and 7<sup>45,67</sup> as well as  
323 with VEGFA, TGF- $\beta$  and PDGF: if any of those domains are deleted, the interaction with the  
324 TGF- $\beta$  superfamily members is disrupted<sup>62</sup>. VEGFA, TGF- $\beta$ s and PDGF have an important role  
325 in pterygium formation<sup>3,68</sup>: an altered interaction of those factors with CRIM1 VWFC domains  
326 could therefore have a role in pterygium development. Sanger sequencing analysis was  
327 subsequently performed for all the six VWFC domains of *CRIM1* (primers listed in  
328 Supplementary Table2) in genomic DNA obtained from two ethnically different populations: 12  
329 affected individuals from Northern Ireland and 9 from Bolivia (Table3).

330 While the H412P variant was found in none of the pterygium affected individuals, one patient  
331 from Bolivia, B1 (Table3), presented with a cytosine to thymine transition in the first position of  
332 codon 745, predicted to result in an Arginine to Cysteine (R745C) amino acid change (dbSNP:  
333 rs145721446) between VWFC-4 and VWFC-5 (exon 13). Although the MAF of this variant is  
334 0.000008237 (Supplementary Fig.1A) and the R745C variant is predicted to be possibly  
335 damaging by PolyPhen, this arginine residue is not conserved in other species and is substituted  
336 by histidine in cattle, mouse, and rat, and by serine in both frog and zebrafish (Supplementary  
337 Fig.1B). The significance of this sequence variant is unclear and not explored here.

338

### 339 **3.3 *CRIM1* expression analysis**

340 Global expression databases indicated *CRIM1* expression in the whole eye, without differentiating  
341 between specific tissues. The location of *CRIM1* expression in pterygium tissue and normal  
342 conjunctiva from sporadic Northern Irish individuals was therefore assessed by  
343 immunohistochemistry (IHC) and qRT-PCR (Fig.3).

344 *CRIM1* was detected, by IHC, lengthwise across the whole pterygium tissue: from the anterior  
345 head (Fig.3A) to the posterior tail (Fig.3C). Moreover, *CRIM1* was observed both in the external  
346 hypertrophic conjunctival epithelium and in the internal fibroblasts, in particular in the vascular  
347 endothelial cells surrounding the blood vessels (Fig.3C arrows).

348 Within the pterygium stroma, *CRIM1* was found around unusual structures such as hair follicles  
349 and inside sebaceous glands (Fig.3B). Finally, *CRIM1* was detected in normal conjunctiva  
350 (Fig.3E) and in post-surgical impression cytology samples of unaffected individuals (Fig.3F).

351 *CRIM1* expression level was further investigated using qRT-PCR on RNA obtained from post-  
352 surgical impression cytology samples (Fig.3F) of unrelated Northern Irish (4 pterygium and 4  
353 controls) individuals, together with a sample from a Northern Irish affected family member (II.2),  
354 found with the *CRIM1* H412P variant (Fig.3G).

355 *CRIM1* expression is significantly higher in pterygium tissues when compared to unaffected  
356 conjunctival controls ( $2^{-\Delta Ct}$  mean values are  $4.22 \pm 0.76$  and  $1.28 \pm 0.122$ , while median values  
357 are 3.4 and 1.3 respectively,  $p = 0.028$ ). Intriguingly, *CRIM1* expression in the Northern Irish  
358 family member II.2 pterygium is lower than that of the all unaffected controls from NI ( $2^{-\Delta Ct}$  value  
359 of 0.32).

360 Thus, we have shown that *CRIM1* expression, both at the RNA and protein level, is higher in  
361 pterygium than in unaffected control tissue.

362

### 363 **3.4 Effects of UV on *CRIM1* expression in corneal epithelial (HCE-S) cells**

364 UV radiation is considered the main epidemiologic factor responsible for pterygium and  
365 pinguecula development<sup>9-11</sup>. Although some papers state that pterygium originates from  
366 conjunctiva and Tenon's fibroblasts<sup>69</sup>, the common consensus is that the first trigger for pterygium  
367 development occurs at the limbal area<sup>1,2,70-72</sup> and pterygium growth has often been described as a  
368 localised limbal stem cell deficiency<sup>70</sup>. Since the aim of this paper is studying the early events  
369 triggering pterygium development, we considered that the best model would be a cell line coming  
370 from the zone where the initial events characterizing the disease have been described: the limbus.  
371 For this reason, for *in vitro* experiments we chose the HCE-S cell line, which spontaneously  
372 originated from corneal cells of the limbal area<sup>44</sup>.

373 The effects of UV light exposure upon HCE-S cells *in vitro* were therefore investigated. The  
374 average daily dose of UV light has been estimated to be 60-70 J/cm<sup>2</sup> in central Europe<sup>74</sup>.  
375 Considering one hour exposure daily in general population, we irradiated an HCE-S monolayer  
376 to a dose of 5.4 J/cm<sup>2</sup> UVA<sup>20,75</sup>, and a dose of 0.5 J/cm<sup>2</sup> UVB.

377 Following UVA exposure, *CRIM1* expression started increasing from 3 hours, becoming  
378 significantly different from untreated control by 6 hours and continuing up to 24 hours ( $2^{-\Delta\Delta Ct} \pm$   
379 SEM values at 3, 6 and 24 hours are respectively:  $0.90 \pm 0.15$ ,  $8.59 \pm 0.22$   $p \leq 0.05$  and  $10.39 \pm$

380 0.40  $p \leq 0.01$ ) (Fig.4A). UVB resulted in a significant increase in *CRIMI* expression at 24 hours  
381 after the treatment when compared to the untreated control ( $2^{-\Delta\Delta Ct} \pm SEM$  values at 3, 6 and 24  
382 hours are respectively:  $1.31 \pm 0.03$ ,  $0.90 \pm 0.01$  and  $1.95 \pm 0.008$ , the last with a  $p \leq 0.05$ ). Since  
383 irradiation with UVA has similar, but greater effects on *CRIMI* expression than UVB, subsequent  
384 experiments were conducted using UVA alone.

385 ERK phosphorylation, previously shown to be increased in pterygium cells following UVA  
386 exposure<sup>19,20</sup>, was assessed in UVA-treated HCE-S cells as described above. This resulted in a  
387 5.6-fold increase in ERK phosphorylation compared to the untreated control at 6 hours after UVA  
388 treatment, rising to 32.1-fold higher at 24 hours (Fig.4B). Treatment of HCE-S cells with the  
389 MEK1/2 inhibitor, U0126, prior to UVA irradiation resulted in a complete inhibition of UVA-  
390 induced ERK phosphorylation.

391 Since UVA significantly elevated both *CRIMI* expression and ERK phosphorylation at 24  
392 hours after treatment, the effects of inhibition of ERK phosphorylation on *CRIMI* expression  
393 following UV irradiation were studied. Surprisingly, inhibition of ERK phosphorylation in HCE-  
394 S cells significantly ( $p < 0.001$ ) potentiated the UV-induced increase in *CRIMI* expression at 24  
395 hours (UVA + inhibitor:  $3.75 \pm 0.12$ , UVA only:  $1.82 \pm 0.15$ , inhibitor only  $0.77 \pm 0.02$ , untreated  
396 control:  $0.63 \pm 0.07$ ; values expressed as  $2^{-\Delta Ct}$ ) (Fig.4C).

397 Several studies have shown that increased ERK activity is associated with a decreased  
398 expression of the anti-apoptotic transcription factor, *BCL2* and an increased apoptosis<sup>76</sup>.  
399 Increased expression of *BCL2* and decreased expression of apoptosis markers has been observed  
400 in pterygia<sup>1,77</sup>. With this in mind, the expression level of *BCL2* was investigated following UVA  
401 treatment activating the ERK pathway in HCE-S cells.

402 Either UVA treatment or inhibition of ERK phosphorylation in HCE-S cells alone significantly  
403 decreased *BCL2* expression ( $2^{-\Delta\Delta Ct} \pm SEM$  values for ERK-I:  $0.53 \pm 0.05$  and UVA  $0.48 \pm 0.06$ ;  
404 both  $p \leq 0.05$ ) (Fig.4D). In comparison to those, when HCE-S cells were treated with MEK1/2

405 inhibitor, U0126, and UVA irradiation, *BCL2* expression was significantly increased ( $2^{-\Delta\Delta C_t} \pm$   
406 SEM of  $1.45 \pm 0.13$ ;  $p \leq 0.001$ ) and restored levels seen in untreated cells ( $p = 0.06$ ).  
407 UV irradiation has therefore a fundamental role in triggering a series of events, including the  
408 increase of *CRIM1* expression and the phosphorylation of ERK, corresponding to a decrease in  
409 *BCL2* levels in HCE-S cells.

410

### 411 **3.5 *CRIM1* intracellular pathway triggered by UVA**

412 *CRIM1*, ERK and *BCL2*, as shown above, are interrelated in playing a pivotal role in the  
413 intracellular pathway triggered by UV exposure in HCE-S.

414 To further investigate the relationship between those factors, we sought to assess the effects of  
415 knocking down expression of *CRIM1* following siRNA transfection. Four siRNAs targeting  
416 *CRIM1*, both separately and in a pool, efficiently knocked down endogenous *CRIM1* expression  
417 in HCE-S cells to less than 30% of untreated level, 48 hours after transfection ( $p \leq 0.001$ )  
418 (Supplementary Fig.2).

419 Endogenous levels of *CRIM1* expression in HCE-S, normalised to cells transfected with NSC  
420 siRNA, increased after UVA treatment by  $1.7 \pm 0.05$  fold,  $p \leq 0.05$ . Because levels of *CRIM1*  
421 expression appear critical and finely regulated following UV irradiation, the concentration of  
422 siCRIM1 was titrated (0.2nM, 0.5nM, 1nM and 10nM) to establish the concentration at which  
423 *CRIM1* expression was the same as non-UVA treated, NSC4-transfected HCE-S cells  
424 (Supplementary Fig.3). The siCRIM1 concentration at which this was achieved was 0.5nM  
425 (Fig.5A).

426 Since pterygium is considered a proliferative rather than degenerative condition<sup>4,70</sup> and UV light  
427 alters cell proliferation at the limbal area as the first event promoting pterygium formation<sup>78</sup>, an  
428 MTT proliferation assay was then performed on HCE-S cells after UVA treatment (Fig.5B). UVA  
429 significantly increased HCE-S proliferation at 72 hours ( $\Delta$ absorbance at 72-24 hours, control

430 0.53 ± 0.02 OD vs UVA 0.6 ± 0.03 OD; p ≤ 0.05). When cells were transfected with 0.5nM  
431 siCRIM1 prior to UVA treatment, HCE-S proliferation was further increased relative to UVA  
432 treatment alone (Δabsorbance at 72-24 hours: 0.7 ± 0.02 OD, with p ≤ 0.05 compared to UVA  
433 and p ≤ 0.01 compared to the mock NSC4 control).

434 As previously shown (Fig.4B), ERK phosphorylation was significantly increased 24 hours  
435 after UV exposure. Transfection of cells with 0.5nM siCRIM1 prior to UV exposure almost  
436 completely abolished the increase in ERK phosphorylation (Fig.5C).

437 Finally, ERK and *CRIM1* regulation were related to *BCL2* expression levels (Fig.5D), which  
438 decreased significantly 24 hours after UVA treatment of HCE-S cells when compared with the  
439 untreated control ( $2^{-\Delta\Delta Ct}$  values for UVA treated HCE-S: 0.61 ± 0.06, p ≤ 0.01). However, prior  
440 transfection with 0.5nM siCRIM1 prevented the decrease in *BCL2* expression, and was not  
441 significantly different from mock NSC4 transfected cells ( $2^{-\Delta\Delta Ct}$  values of siCRIM1+UVA: 0.96  
442 ± 0.08).

443 The latest results obtained using siCRIM1 were able not only to confirm the previous data  
444 showing a UVA-mediated increase in *CRIM1* expression, ERK phosphorylation and a decrease  
445 in *BCL2* expression, but also to demonstrate that by modulating only *CRIM1* expression, the  
446 whole signalling pathway leading to cell proliferation in response to UVA exposure can be  
447 regulated.

448

### 449 **3.6 Effects of wild type and H412P mutant *CRIM1* over-expression in HCE-S cells**

450 Based on the outlined pathway involving *CRIM1* regulation upon UV exposed HCE-S cells, the  
451 consequences of the H412P *CRIM1* variant in a pterygium pathogenic context were investigated.  
452 The H412P variant was introduced into the human *CRIM1* sequence by site directed mutagenesis  
453 and the complete *CRIM1* sequence checked by Sanger sequencing (Supplementary Table1),

454 confirming the presence of the c.1235 A>C substitution and absence of any other variation in the  
455 sequence.

456 HCE-S cells were transfected with an empty plasmid, wild type or the *CRIMI*:p.H412P  
457 expression plasmid. A significant *CRIMI* overexpression with respect to endogenous *CRIMI* was  
458 revealed by qRT-PCR both at 48 hours (*CRIMI* wt  $6 \pm 0.82$ ,  $p < 0.01$  and *CRIMI* H412P  $5.8 \pm$   
459  $0.97$ ,  $p < 0.05$ ) and at 72 hours (*CRIMI* wt  $6.4 \pm 1.075$ ,  $p < 0.01$  and *CRIMI* H412P  $6.3 \pm 1.4$   
460  $p < 0.01$ ) after transfection (Fig.6A).

461 Since a decrease in proliferation was reported in vascular endothelial cells after transient *CRIMI*  
462 overexpression<sup>54</sup>, an *in vitro* MTT proliferation assay was then performed in HCE-S cells  
463 transfected with *CRIMI* wild type or H412P constructs (Fig.6B). Compared to mock transfected  
464 HCE-S cells, wild type *CRIMI* overexpression had a relevant anti-proliferative effect, which was  
465 most significant at 72 hours post-transfection ( $\Delta$ absorbance 72-24 hours  $0.60 \pm 0.02$  OD;  $p < 0.01$ ).  
466 This effect was not observed in the *CRIMI* H412P transfected cells ( $\Delta$ absorbance 72-24 hours  
467  $0.52 \pm 0.02$  OD), which did not differ significantly from the mock transfected control  
468 ( $\Delta$ absorbance 72-24 hours  $0.57 \pm 0.01$  OD).

469 ERK phosphorylation is increased, simultaneously with *CRIMI* expression, in vascular  
470 endothelial cells after VEGFA treatment<sup>79</sup>. Here, ERK phosphorylation was strongly increased in  
471 the wild type *CRIMI* transfected HCE-S compared to mock transfected control and to H412P  
472 *CRIMI* transfected cells at 72 hours post-transfection (Fig.6C).

473 Other gene expression levels, possibly affected by *CRIMI* H412P variant, were investigated  
474 by qRT-PCR: *VEGFA* for its role in pterygium angiogenesis<sup>3,72</sup> and its direct interaction with  
475 *CRIMI*<sup>73</sup>, Transforming Growth Factor beta I (*TGF- $\beta$ I*) for its involvement in several other eye  
476 diseases<sup>80</sup> and *BCL2* for its importance in apoptosis, previously documented in pterygium<sup>77,81</sup>.  
477 HCE-S cells were transfected with wild type and H412P *CRIMI* plasmids and gene expression  
478 was assessed at 48 and 72 hours after transfection (Fig.6D). Levels of *VEGFA* and *TGF- $\beta$ I*

479 expression were not significantly different between the wild type and the H412P *CRIMI*  
480 transfected cells (*VEGFA*: wild type 48 hours  $0.8351 \pm 0.0740$ , H412P 48 hours  $0.8966 \pm 0.0630$ ,  
481 wild type 72 hours  $1.1447 \pm 0.0940$ , H412P 72 h  $1.0443 \pm 0.1100$ , *TGF- $\beta$ I*: wild type 48h  $0.7410$   
482  $\pm 0.0730$ , H412P 48h  $0.8630 \pm 0.0480$ , wild type 72h  $0.9428 \pm 0.0650$ , H412P 72h  $1.1810 \pm$   
483  $0.1080$ , all values are expressed in  $2^{-\Delta C_t}$ ). In contrast, a significant decrease in *BCL2* expression  
484 level was observed in the wild type *CRIMI* transfected cells with respect to H412P mutant and  
485 mock transfected cells (p value <0.05) both at 48 and 72 hours (wild type 48 hours:  $0.5453 \pm$   
486  $0.0720$ , H412P 48 hours:  $1.1647 \pm 0.1800$  and wild type 72 hours:  $0.5977 \pm 0.0240$ , H412P 72  
487 hours:  $1.2376 \pm 0.1350$ , all values are expressed in  $2^{-\Delta C_t}$ ), suggesting that overexpression of  
488 *CRIMI* may play a role in up-regulation of apoptosis, which is normally induced in cornea and  
489 conjunctiva by UV radiation<sup>82,83</sup>.

490 The effect of increased *CRIMI* expression upon apoptosis in HCE-S cells was investigated in  
491 *CRIMI* transfected cells by TUNEL assay (Fig.6E). At 72 hours after transfection the wild type  
492 *CRIMI* transfected HCE-S cells showed a significantly higher rate of apoptosis compared to either  
493 the H412P *CRIMI* or mock transfected HCE-S. The percentage of apoptotic (TUNEL positive),  
494 wild type *CRIMI* transfected cells ( $25.6 \pm 1.8$ ) was significantly higher than either H412P *CRIMI*  
495 plasmid or mock transfected HCE-S cells ( $3.9 \pm 0.4$  and  $2.7 \pm 0.5$  respectively;  $p < 0.001$ ) (Fig.6E).  
496 We have shown here that *CRIMI* plasmid overexpression exerts the same effects of UV induced  
497 *CRIMI* increased intracellular levels, in terms of decreased cell proliferation, increase in ERK  
498 phosphorylation and reduction in *BCL2* expression, followed by a higher rate of apoptosis.  
499 The presence of H412P variant in *CRIMI* however abolished any of those effects, supporting a  
500 role for this variant in the increased susceptibility of members of the Northern Irish family to  
501 pterygium and pinguecula.

502

503 **4. DISCUSSION**

504 UV light presents a chronic stimulus to the eye surface, altering the normal processes of growth  
505 control in cornea and conjunctiva, and is associated with several pathologies affecting the anterior  
506 eye<sup>84</sup> such as photokeratitis<sup>85</sup>, climatic droplet keratopathy<sup>10</sup>, cortical cataract<sup>86</sup>, squamous cell  
507 carcinoma<sup>87</sup> and pterygium<sup>9,21</sup>. Pterygium, resulting in 1% of all the ocular surgeries in developed  
508 countries<sup>88</sup> and presenting a 12% surgery recurrence rate<sup>5</sup>, represents a substantial cost to National  
509 Health Services and the community, estimated in Australia as US \$100M per year<sup>89</sup>. In the  
510 identified Northern Irish family (Fig.1), affected by pterygium or pinguecula despite not being  
511 exposed to high levels of sunlight, genetic predisposition may play a fundamental role in the  
512 etiologic process (Table 1). A WES approach to candidate gene identification with *in silico* and  
513 literature analysis led to selection of H412P variant in *CRIM1* gene for several reasons. Firstly,  
514 the substitution of a highly conserved, positively charged histidine (H) residue with an apolar  
515 proline (P) is predicted to interfere with CRIM1 structure and function.

516 Moreover, the interaction between F1 domains of fibronectin, structurally similar to VWF  
517 domains of CRIM1 where H412P is located, and VEGF is enhanced at acidic pH (5.5-7)<sup>66</sup>. As  
518 Histidine works at this pH range (pKa<sub>3</sub>=6), a mutation in this residue may interfere with the  
519 electrostatic binding of other ligands.

520 CRIM1's VWF domains (Fig.2) were also shown essential for interaction with VEGFA<sup>62</sup>, the  
521 angiogenic factor overexpressed in the vascularised pterygium tissue following UV exposure<sup>3,72</sup>.  
522 CRIM1 is thus proposed as a VEGFA antagonist<sup>60,73</sup>, possibly resisting pterygium formation by  
523 preventing angiogenesis as it has been shown to prevent proliferation<sup>54,55</sup>. Proliferation in  
524 cardiomyocytes is reduced by CRIM1 upregulation following miR199a silencing<sup>55,56</sup>, the same  
525 miRNA which was found overexpressed in pterygium<sup>90</sup>. In addition to proliferation and  
526 angiogenesis, tissue remodelling through cell adhesion and migration also occurs during  
527 pterygium formation<sup>4</sup>. This process has been shown to be regulated by CRIM1 in neurons<sup>57</sup>, lung

528 cancer<sup>59</sup> and lens epithelial cells<sup>58</sup>, the latter showing a premature fibre differentiation when  
529 *CRIMI* is lost<sup>91</sup>.

530 As previously described, pterygium etiopathogenesis is generally ascribed to UV eye exposure  
531 and *CRIMI* was found to be involved in UV related diseases such as bovine eye cancer<sup>63</sup> and  
532 melanoma<sup>64</sup>. Finally, recent studies have elucidated the importance of *CRIMI* expression during  
533 the anterior eye development<sup>50,51,58</sup>.

534 *CRIMI* was thus selected as our best candidate responsible for pterygium pathogenesis within  
535 the NI family. However, a reduced penetrance, as previously described in pterygium<sup>1,26,31</sup>, makes  
536 interpretation of genetic data challenging<sup>36</sup>. While two unaffected members of the NI family (III.2  
537 and III.3) harbour the H412P variant in *CRIM1*, no affected member was found that did not have  
538 the H412P variant. Moreover, given the younger age of the unaffected members (34 and 48 years  
539 old), penetrance in this case could be age-related, as described in MEN1 syndrome, in which  
540 tumour occurrence increases with the age, becoming fully penetrant only after sixty years<sup>92</sup>. It is  
541 therefore possible that III.2 and III.3 family members may never develop pterygium or will do so  
542 later in their lifetime; in any case, they should be monitored closely for development of pterygium  
543 in the future.

544 Neither the *CRIMI* H412P variant nor any other alteration within the six VWFC domains of  
545 *CRIMI* were found in other affected unrelated individuals from Northern Ireland.

546 Only one other patient from Bolivia was identified with a missense variant located between  
547 VWFC-4 and VWFC-5: R745C (Supplementary Fig.1). While this arginine residue is not  
548 conserved between the species, the introduction of a cysteine residue may facilitate disulphide  
549 bridge formation with highly conserved cysteine residues in the previous or subsequent VWFC  
550 domain, vital to the VWF domain's structure<sup>45,93</sup>.

551 While no variants in *CRIMI* were found in the NI patients, *CRIM1* expression was elevated  
552 throughout the pterygium tissue compared to unaffected conjunctiva (Fig.3). In particular, *CRIMI*  
553 expression was observed around pterygium blood vessels which, together with its previously

554 described expression in endothelial cells during capillary formation<sup>60,61</sup> and its interaction with  
555 VEGFA<sup>73</sup>, suggests a role for CRIM1 during the critical angiogenic processes of pterygium  
556 formation<sup>1,21</sup>. CRIM1 was also detected in unusual structures such as hair follicles and sebaceous  
557 glands, previously identified as characteristic features of some pterygia<sup>70</sup>. These structures are  
558 representative of an uncontrolled transdifferentiation program<sup>94</sup> occurring in pterygium in  
559 addition to an epithelial-mesenchymal transition (EMT) or fibrosis process<sup>95</sup>, and likely reflects  
560 similarities between pterygia, limbal dermoids and hair epithelia<sup>96</sup>.

561 Comparing *CRIM1* expression levels between pterygium-affected and -unaffected individuals,  
562 those were significantly increased in the affected samples (Fig.3G), while the lowest *CRIM1*  
563 expression was recorded for the affected family member (II.2). A high *CRIM1* expression in  
564 pterygium-affected individuals could therefore represent a defensive cellular response mechanism  
565 against UV damaging effects, which is impaired by the H412P variant within the NI family.

566  
567 Involvement of CRIM1 in UV-related diseases either in the eye<sup>63</sup> or the skin<sup>64</sup> is not surprising  
568 considering that UV irradiation affects the balance between proliferation and apoptosis in several  
569 cell types, including skin keratinocytes<sup>97,98</sup>, corneal epithelium<sup>82</sup> and pterygium basal epithelial  
570 cells<sup>77</sup>. However, while the eye is exposed to both UVA and UVB radiation, the intensity of UVA  
571 radiation reaching the Earth's surface has 10-100 times the intensity of UVB<sup>99</sup> and it is known to  
572 induce oxidative stress in exposed cells and tissues, including pterygium<sup>100</sup>. Accordingly, UVA  
573 induced a more rapid and larger response than UVB in HCE-S cells (Fig.4A).

574 Exposure of HCE-S cells to UVA radiation leads to an increase in *CRIM1* (Fig.4A) expression.  
575 The elevated ERK phosphorylation upon UVA irradiation we observed (Fig.4B) was previously  
576 described in UVA or UVB irradiated pterygium and conjunctival cells<sup>19,20</sup>. While inhibition of  
577 ERK phosphorylation alone had no effect on the expression of *CRIM1*, it potentiated the *CRIM1*  
578 up-regulation when HCE-S cells were treated with UV (Fig.4C). This underscores the importance  
579 of UV irradiation as the primary trigger for the intracellular pathway but underlines also the

580 capacity of ERK phosphorylation to regulate *CRIMI* expression levels. Moreover, inhibition of  
581 ERK phosphorylation in HCE-S cells treated with UV abolished the UV-induced decrease in  
582 *BCL2* expression (Fig.4D): UV decreases *BCL2* levels through ERK phosphorylation, which  
583 results therefore in upstream *BCL2* regulation within this pathway.

584 The role of *CRIMI* in the cellular response to UV exposure was confirmed in HCE-S cells in  
585 which *CRIMI* over-expression induced by UV treatment was inhibited by siRNA transfection  
586 (Fig.5A). An increased cell proliferation in siCRIM1-treated cells upon UV exposure (Fig.5B),  
587 suggests that *CRIMI* has a role in protecting against pterygium formation by minimising cell  
588 proliferation in response to UV radiation. When siCRIM1 was added to UV treatment, HCE-S  
589 cells decreased ERK phosphorylation to that seen in untreated cells (Fig.5C) and restored *BCL2*  
590 basal expression (Fig.5D), revealing that also *CRIMI* is able to control ERK phosphorylation as  
591 well as *BCL2* expression and locates it upstream of those two factors within the pathway. Those  
592 experiments showed how *CRIMI* exerts a central role within the UV triggered intracellular  
593 pathway and silencing its expression is enough to counter all those alterations we examined.

594 Previous studies have shown how *CRIMI* alterations influences cellular function: multiple exon  
595 deletions of *CRIMI* lead to syndromic disease<sup>52</sup> while a minor alternately spliced *Crim1* isoform  
596 determines perinatal lethality with multiple organ dysfunction<sup>51</sup>; however, the effects of *CRIMI*  
597 missense mutations still remain unexplored. Therefore, based on the intracellular mechanism  
598 described, the effects of the H412P variant found within the Northern Irish kindred were studied.  
599 *CRIMI* over-expression in HCE-S cells leads to reduced proliferation (Fig.6B) and a parallel  
600 increase in apoptosis (Fig.6D), as expected following the previous results where *CRIMI* was  
601 shown having a protective role in pterygium formation, counteracting the UV-induced  
602 proliferation (Fig.4B). This is in accordance with the downregulation of vascular endothelial cell  
603 proliferation after transient *CRIMI* overexpression<sup>54</sup> and with the increase in cardiomyocyte  
604 proliferation following *CRIMI* silencing through miR-199a<sup>55,56</sup>. Since the same miR-199a was  
605 found upregulated in pterygium<sup>90</sup>, this could analogously increase cell proliferation in pterygium

606 formation through *CRIM1* silencing, counteracting the protective effects of *CRIM1*  
607 overexpression.

608 *CRIM1* overexpression was also able to increase ERK phosphorylation (Fig.6B) following UVA  
609 irradiation (Fig.4B). This result confirms previous data (Fig.5C) showing that the effects of  
610 *CRIM1* overexpression lie upstream of ERK phosphorylation and demonstrates that it does not  
611 need UV to exert its function.

612 All the effects of *CRIM1* overexpression in HCE-S cells were almost completely abolished by the  
613 H412P variant in *CRIM1* (Fig.6).

614 The results obtained delineated a finely regulated pathway where either UV (Figure 4B) or  
615 elevated CRIM1 levels (Figure 6C) increase ERK phosphorylation, demonstrating that CRIM1  
616 lies upstream of, and is responsible for ERK phosphorylation.

617 CRIM1 expression was increased upon UV exposure and this increase was potentiated when the  
618 U0126 inhibitor was added to the UV-irradiated cells. This suggests a feedback mechanism in  
619 which ERK phosphorylation, increased by elevated *CRIM1* levels, serves to limit the rise in  
620 *CRIM1* expression triggered by UV (Fig.7). UV is therefore necessary for the activation of the  
621 whole pathway because the U0126 inhibitor alone, without UV treatment, does not increase  
622 CRIM1 expression (Figure 4C). For the first time an increase in *CRIM1* expression has been  
623 directly correlated with a consequent activation of the ERK pathway in corneal cells.

624 Based on the *in vitro* experimental evidence and on the feedback mechanism outlined, H412P can  
625 be considered either a loss-of-function or a dominant-negative mutation. Similarly, a study on a  
626 shortened mouse *Crim1* isoform was unable to conclude whether the mechanism was  
627 hypomorphic (reduced activity) or dominant negative<sup>51</sup>, although the latter have been described  
628 in growth factor receptors which bind the same CRIM1 interactors such as PDGF<sup>101</sup> or VEGF<sup>102</sup>  
629 and in VWF domains similar to those found in CRIM1<sup>103,104</sup>.

630

631

632 **5. CONCLUSIONS**

633 In summary, we have shown that *CRIMI* plays an important role in the response of corneal  
634 cells to UV irradiation by activation of a finely regulated intracellular pathway where increased  
635 *CRIMI* expression counteracts cell proliferation and increases apoptosis. We demonstrate that  
636 *CRIMI* H412P variant impairs this response, suggesting its involvement in the increased  
637 susceptibility to pterygium observed in a Northern Irish family. Further investigation of the role  
638 of CRIM1 in UV triggered pathways and pterygium holds promise as a target for the prevention  
639 and treatment of development or recurrence of pterygium or other UV related diseases.

640

641

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644 contributions to this research.

645 The WES investigation was supported by the NIHR Biomedical Research Centre, Oxford.

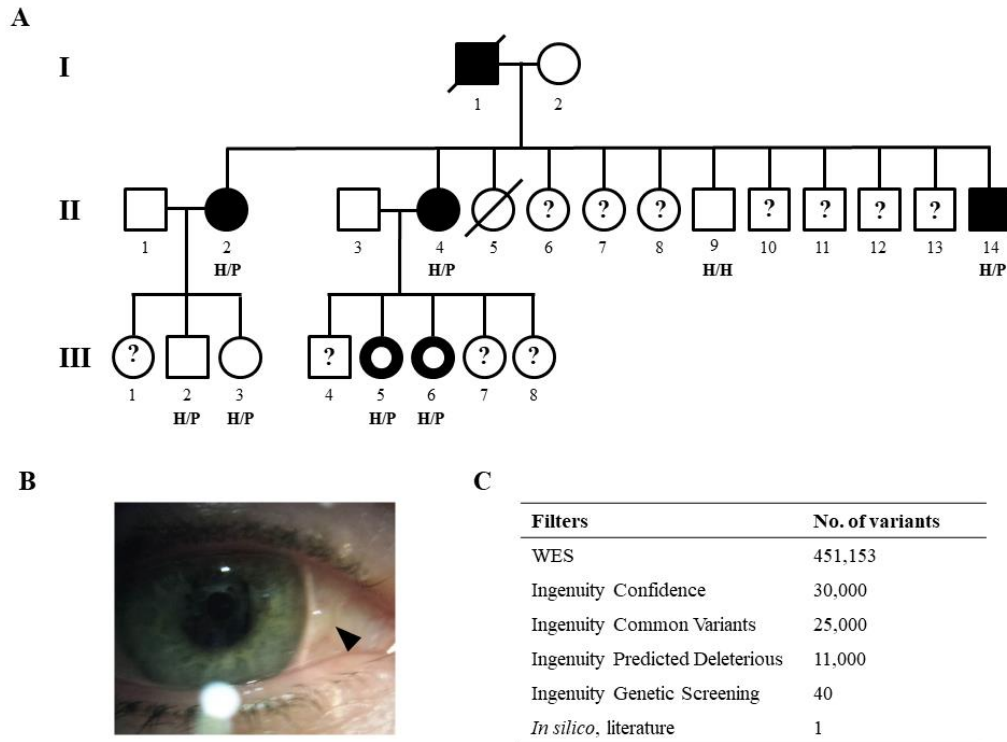
646 **Conflict of Interest Statement** None declared.

647 **Funding:** Ulster University Vice-Chancellor's Research Scholarships (VCRS)

648

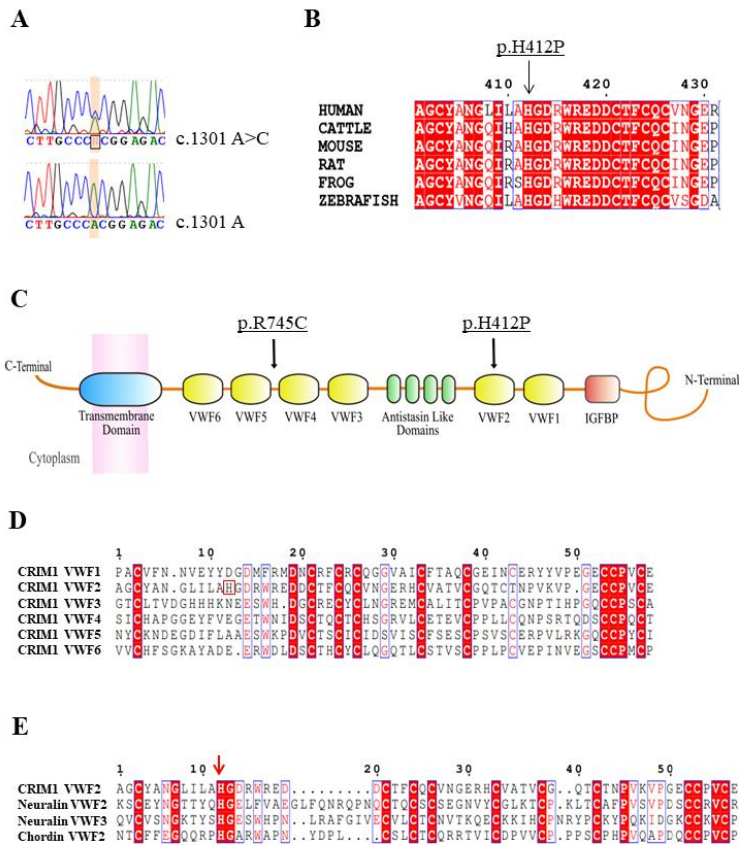
649

## 7. FIGURE LEGENDS



651

652 **Figure 1.** Northern Irish family affected by pterygium or pinguecula and WES analysis. **(A).** Pedigree  
 653 of a Northern Irish family affected with pterygium. Open symbols denote unaffected individuals;  
 654 filled black symbols denote pterygium affected individuals and filled black symbols with open circles  
 655 inside denote pinguecula affected individuals. Squares represent male and circles represent female  
 656 individuals. Slashed symbols denote deceased family members and question marks are for individuals  
 657 who have not participated in the study. Although all family members were invited to participate in  
 658 this study, only those who gave informed consent were enrolled. **(B).** Pinguecula visible at the limbal  
 659 area, between the cornea and conjunctiva (arrowhead). The image was obtained from family member  
 660 III:5. **(C).** WES variants screening. Ingenuity variant analysis screened the 451,153 variants obtained  
 661 with WES using four subsequent filters: Confidence to ensure quality, Common Variants (MAF <  
 662 0.05), Predicted deleterious using Polyphen and SIFT and Genetic, assuming an autosomal dominant  
 663 inheritance pattern. A final *In silico* analysis based on the residue conservation through BLAST and  
 664 Clustal Omega, expression analysis using TiGER database and literature research allowed selection  
 665 of a single candidate variant in *CRIMI* gene.



666

667 **Figure 2.** CRIM1 (Cysteine RIch transMembrane BMP regulator 1) sequence in the NI family,

668 conservation and structure analysis. (A). Electropherogram of Sanger sequencing performed in the

669 family members confirmed the c.1235 A>C heterozygous transversion mutation in affected

670 individuals and which was homozygous for wild-type A allele in the unaffected control. (B). Clustal

671 X2.1 multiple sequence alignment of orthologous CRIM1 protein sequences from Human

672 (NP\_057525.1), Cattle (NP\_001192227.1), Mouse (NP\_056615.1), Rat (NP\_001162574.1), Frog

673 (NP\_001163917.1), Zebrafish (NP\_997986.1) indicates that the H412P missense mutation occurs in

674 a highly conserved residue. EsPript 3.0. Legend: Red box, white character: strict identity; Red

675 character: similarity in a group; Blue frame: Similarity (C). Schematic ideogram of Human CRIM1,

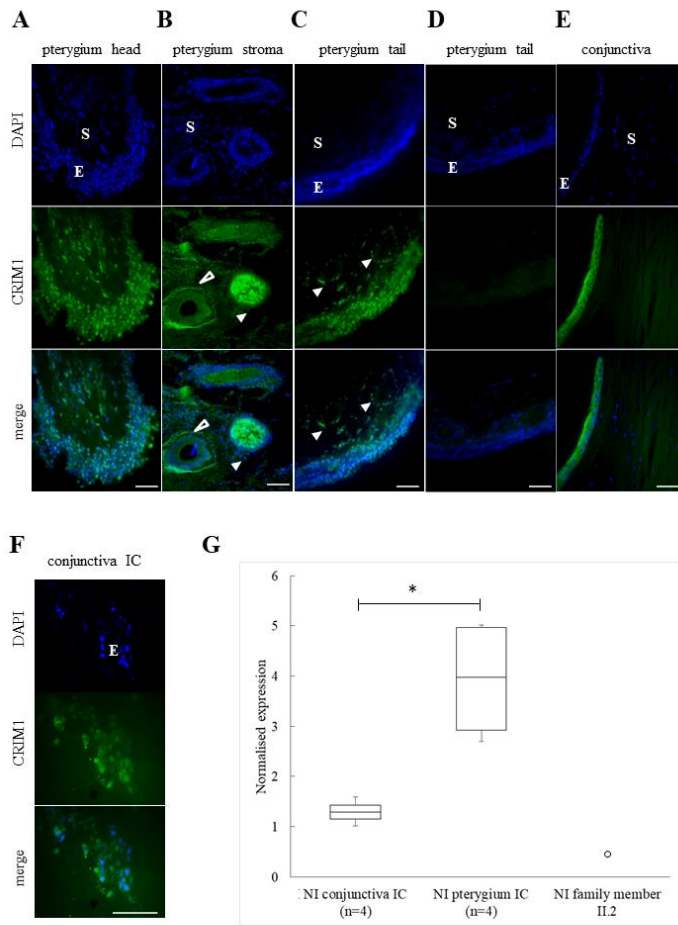
676 characterised by multiple domains: one transmembrane (blue), six von Willebrand factor (VWF)

677 (yellow), four antistatin-like (green) and one IGFBP (red). The position of the H412P and the R745C

678 mutation are indicated. (D). Clustal X2.1 alignment of the six VWFC domains in *CRIM1* shows that,

679 other than in VWFC-2, H412 is not conserved in the other five VWFC domains. (E). Clustal X2.1

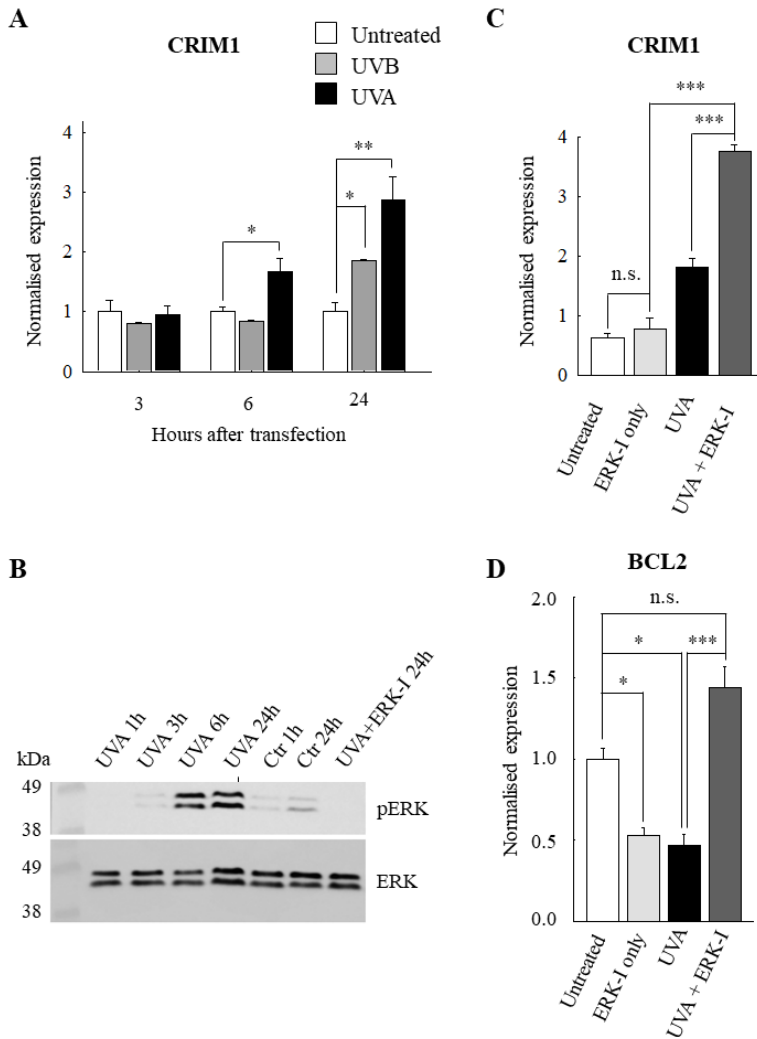
680 alignment of CRIM1 with other BMP antagonists indicates that the residue H412 is conserved in the  
681 VWFC-2 domain of human chordin and in the VWFC-2 and 3 domains of human neuralin.  
682 across groups (if more than 70% of the residues within the frame share similar physico-chemical  
683 properties).  
684



685

686 **Figure 3.** CRIM1 expression analysis in pterygium and conjunctiva from Northern Ireland. **A-F**  
687 images represent immunohistochemical (IHC) staining of CRIM1 expression (green) and nuclei  
688 (DAPI, blue) in pterygium and conjunctival tissues obtained from sporadic Northern Irish patients.  
689 Scale bar, 100µm (**A**). CRIM1 expression in a pterygium head, visible in both the external epithelial  
690 layer (E) as well as the internal stroma (S). (**B**). CRIM1 expression shown in peculiar structures  
691 identified in pterygium stroma: a hair follicle (empty arrowhead) and a sebaceous gland (plain  
692 arrowhead). (**C**). CRIM1 expression in pterygium tail, arrowheads indicate CRIM1 expression in  
693 cells surrounding the blood vessels. (**D**). Negative IgG control in pterygium tail tissue. (**E**). CRIM1  
694 expression in conjunctival epithelial tissue of an unaffected individual. (**F**). IHC of a post-surgical  
695 impression cytology sample obtained from unaffected superficial epithelial conjunctival cells:  
696 CRIM1 is expressed in all the cells captured on the membrane filter. (**G**) *CRIM1* expression is

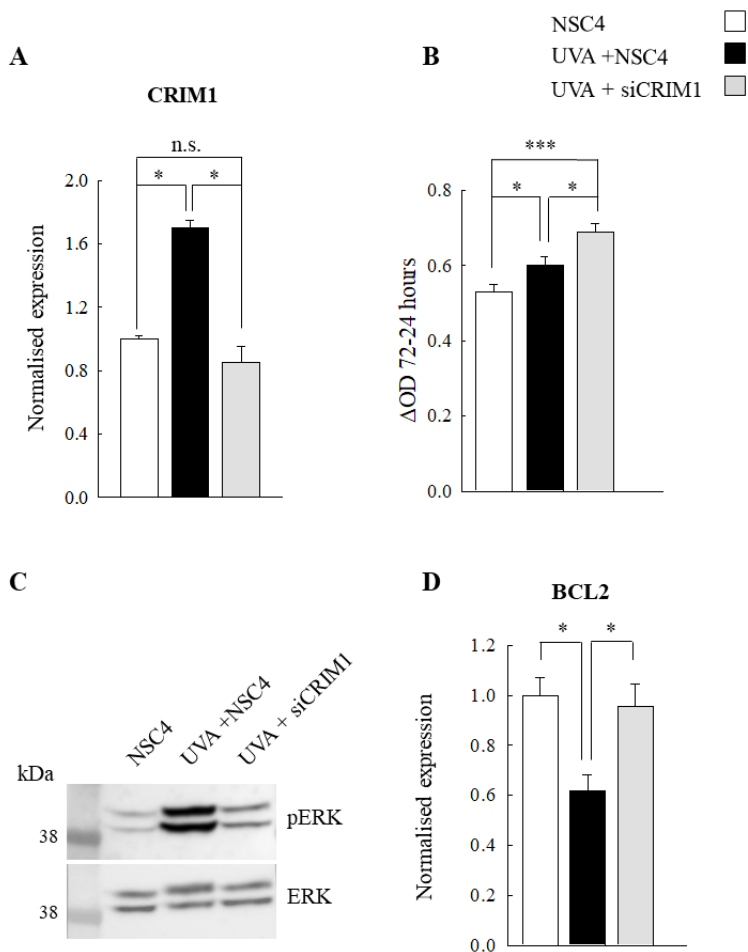
697 increased in pterygium patients from Northern Ireland. qRT-PCR analysis of *CRIMI* carried out in  
698 cDNA from post-surgical impression cytology samples: NI conjunctival individual controls, NI  
699 pterygium individuals and one affected NI family member (II.2 in Fig. 1). All values are expressed  
700 in  $2^{-\Delta C_t} \pm \text{SEM}$ . n=2 with two technical replicates each sample.  
701



702

703 **Figure 4.** UV treatment of HCE-S cells increases CRIM1 expression, ERK phosphorylation and  
 704 decreases BCL2 expression. **(A).** qRT-PCR revealed significantly increased CRIM1 expression levels  
 705 in HCE-S cells at 6 ( $p \leq 0.05$ ) and 24 hours ( $p \leq 0.01$ ) after UVA treatment and at 24 hours ( $p \leq 0.05$ )  
 706 after UVB treatment compared to the untreated control. **(B).** Western blot analysis of HCE-S cell  
 707 lysates revealed an increase in ERK phosphorylation at 6 and 24 hours after UVA irradiation with  
 708 respect to ERK phosphorylation levels of untreated control. Pretreatment of cells with U0126  
 709 inhibitor abolished ERK phosphorylation in UV treated cells. **(C).** qRT-PCR was used to evaluate  
 710 *CRIM1* expression following UV treatment in the presence of MEK1/2 inhibitor, U0126. Inhibition  
 711 of ERK pathway activation enhances the increase in *CRIM1* expression seen in UV irradiated HCE-  
 712 S cells whilst inhibition of ERK phosphorylation alone has no significant effect. **(D).** *BCL2*  
 713 expression measured by qRT-PCR. HCE-S cells were UVA irradiated with and without U0126 and

714 harvested 24 hours after treatment. A significant decrease in *BCL2* expression was observed when  
715 the cells were treated with either U0126 or UVA alone but not in combination when compared to the  
716 HCE-S untreated cells. qRT-PCR data represent fold change of the  $2^{-\Delta\Delta C_t}$  mean  $\pm$  SEM compared to  
717 untreated HCE-S. n=3 with three technical replicates each.  
718



719

720 **Figure 5.** siCRIM1 0.5nM is able to restore normal HCE-S conditions after UVA treatment. **(A).**

721 qRT-PCR shows the amount of siCRIM1 (0.5nM) able to restore endogenous CRIM1 levels in

722 HCE-S after UVA exposure. For all the experiments HCE-S cells were treated with: NSC4, UVA +

723 NSC4 and UVA+ siCRIM1 (0.5nM) and harvested 24 hours later. Data represent  $2^{-\Delta C_t} \pm SEM$ . n=3

724 with three technical replicates for each sample. **(B).** An MTT assay demonstrates an increased

725 proliferation upon UVA exposure, which is further increased if *CRIM1* expression is restored to

726 pre-treatment endogenous levels (siRNA 0.5nM), confirming the antiproliferative effect of CRIM1.

727 n=6 with eight technical replicates for each condition. **(C).** Western Blot analysis shows ERK

728 phosphorylation (pERK) at 24 hours after UVA treatment in HCE-S cells. The increased ERK

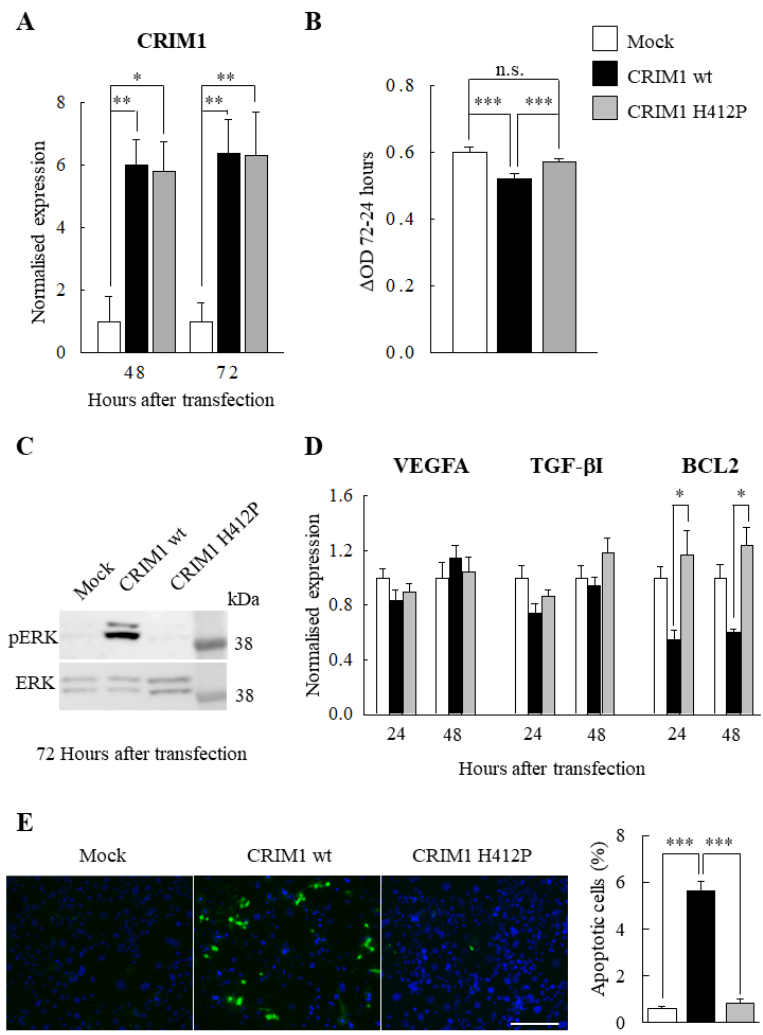
729 phosphorylation due to UVA exposure was brought back to normal levels following transfection

730 with 0.5nM CRIM1 siRNA. **(D).** *BCL2* expression, measured by qRT-PCR, decreases upon UVA

731 irradiation eliciting apoptosis but is restored in HCE-S cells treated with siCRIM1. Data represent 2<sup>-</sup>

732  $\Delta\Delta\text{Ct} \pm \text{SEM}$  with respect to untransfected HCE-S. n=3 with three technical replicates each sample.

733



734

735 **Figure 6.** *CRIM1* wild type overexpression in HCE-S cells results in a decrease in cell proliferation  
 736 and an increase in ERK phosphorylation and apoptosis. (A). qRT-PCR showing *CRIM1*  
 737 overexpression in mRNA obtained from HCE-S cells transfected with Human *CRIM1* wild type and  
 738 mutant (H412P) in pcDNA3.1 expression plasmid. Both *CRIM1* wild type (wt) and H412P mutant  
 739 were significantly overexpressed at 48 and 72 hours after transfection with respect to the mock  
 740 control. Data represent fold change of the  $2^{-\Delta C_t}$  mean  $\pm$  SEM. n=3 with 3 technical replicates for each  
 741 condition. (B). An MTT assay of HCE-S cell proliferation was performed at 72 hours after  
 742 transfection with empty plasmid (mock), *CRIM1* wt and H412P mutant constructs. *CRIM1* wt, when  
 743 overexpressed, has an anti-proliferative effect when compared to the mock transfected control  
 744 ( $p < 0.001$ ). Overexpression of the mutated H412P *CRIM1* does not have the same anti-proliferative

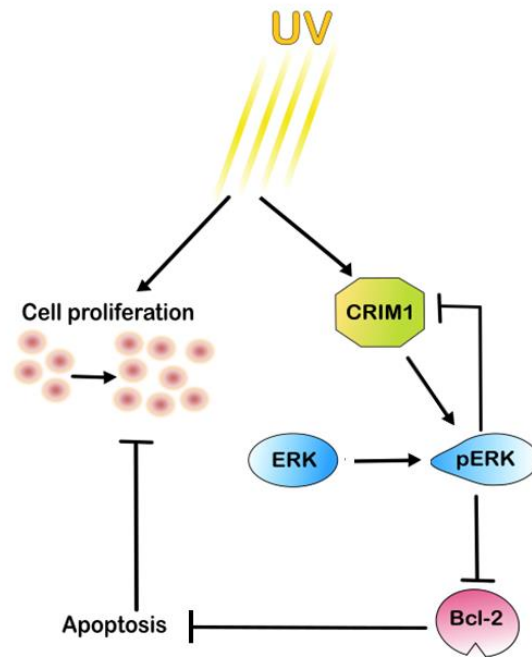
745 effect. n=6 with 8 technical replicates for each condition. **(C)**. ERK phosphorylation (pERK) was  
746 detected by Western Blot analysis at 72 hours post transfection with mock, *CRIMI* wt and H412P  
747 plasmids in HCE-S cells. *CRIMI* wt overexpression resulted in a high level of ERK phosphorylation  
748 in comparison to *CRIMI* H412P and mock control. **(D)**. qRT-PCR analysis of *VEGFA*, *TGF-β1* and  
749 *BCL2* expression in HCE-S cells transfected with mock, *CRIMI* wt and H412P plasmids and  
750 harvested after 48 and 72 hours. No significant variation of *VEGFA* and *TGF-β1* expression was  
751 observed between mock, *CRIMI* wt and *CRIMI* H412P while *BCL2* expression significantly  
752 decreased in H412 wt *CRIMI* transfected cells compared with mock transfected HCE-S both at 48  
753 and 72 hours post transfection. Data represent fold change of the  $2^{-\Delta Ct}$  mean  $\pm$  SEM respect to mock  
754 transfected HCE-S. n=3 with three technical replicates each. **(E)**. TUNEL assay in HCE-S cells  
755 transfected with wt and H412P mutant *CRIMI* plasmids. TUNEL-positive cells are stained green and  
756 nuclei are stained blue with DAPI. Overexpression of *CRIMI* wt results in increased apoptosis  
757 compared to either the *CRIMI* H412P or mock transfected cells. Scale bar, 100  $\mu$ m. TUNEL assay  
758 quantification was performed in 12 fields per condition using ImageJ. n=3

759

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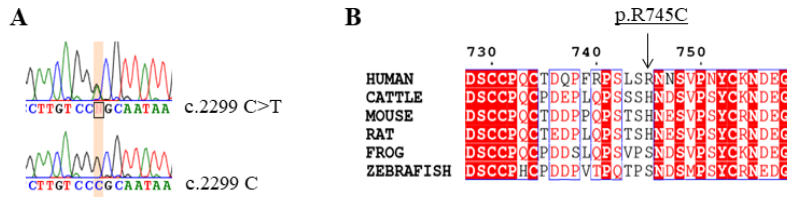
763

764 **Figure 7.** CRIM1 regulates pERK in a looped pathway. Here, based on our experimental evidence,  
 765 we propose, within a schematic, an intracellular pathway triggered by UV that may act as a  
 766 protective mechanism against pterygium development. When cells are exposed to UV light, CRIM1  
 767 expression is increased, which in turn increases ERK phosphorylation. This, in turn, blocks further  
 768 CRIM1 expression in the feedback loop shown. Inhibition of ERK phosphorylation prevents this  
 769 negative feedback, resulting in further increases of CRIM1 expression.

770 ERK phosphorylation induces decreased expression of the anti-apoptotic BCL2, initiating cell  
 771 apoptosis. This proposed pathway was shown to be impaired in the case of the H412P mutation in  
 772 CRIM1, found in the Northern Irish family affected by pterygium.

773

774



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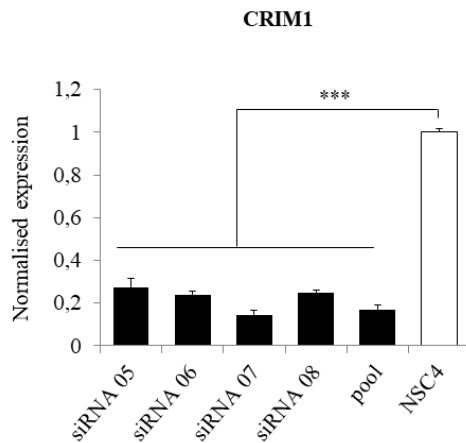
776 **Supplementary Figure 1.** R745C variant found in a pterygium affected patient from Bolivia. **(A).**

777 Electropherogram showing the novel c.2299C>T transition variant from the Bolivian patient (B1),

778 which corresponds to the R745C variant. **(B).** Multiple *CRIMI* sequence alignment shows that the

779 R745 residue is not conserved across species; it is conserved however the absence of cysteine between

780 VWFC-4 (residues 677-735) and VWFC-5 (residues 751-809).



781

782 **Supplementary Figure 2.** siRNAs targeting *CRIMI* efficiently knock down its expression in HCE-

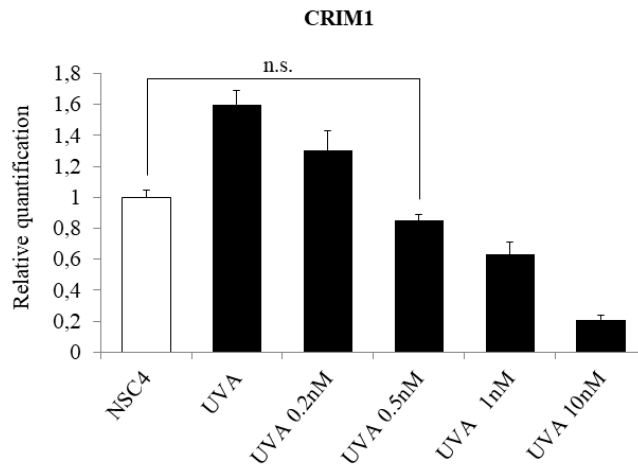
783 S cells. *CRIMI* expression obtained using qRT-PCR of HCE-S cell cDNA 48 hours post transfection.

784 All four siRNAs tested were able to knock down *CRIMI* expression, including the pool of four

785 siRNAs used at the same final concentration of 10 nM. qRT-PCR data represent fold change of the

786  $2^{-\Delta\Delta Ct}$  mean  $\pm$  SEM with respect to untreated HCE-S. n=2 with three technical replicates each.

787



788

789 **Supplementary Figure 3.** A dose response curve shows that 0.5nM siRNA pool restores HCE-S  
 790 endogenous *CRIM1* levels. *CRIM1* expression obtained using qRT-PCR of HCE-S cell cDNA 48  
 791 hours post transfection with UVA and the siRNA pool at different concentrations. qRT-PCR data  
 792 represent fold change of the  $2^{-\Delta\Delta C_t}$  mean  $\pm$  SEM with respect to untreated HCE-S. n=2 with three  
 793 technical replicates each.

794

795 **TABLES**

<b>Family member</b>	<b>Year of birth</b>	<b>Sex</b>	<b>Eye condition</b>	<b>Age of diagnosis</b>	<b>Lived abroad</b>	<b>Time in sunny climates</b>	<b>Wear sunglasses</b>
II.2	1944	F	pterygium	40 years	No	1/year, 3 weeks	Sometimes
II.4	1946	F	pterygium	59 years	Canada 13 years	1/year, 2 weeks	No
II.9	1958	M	unaffected	-	No	1/year, 3 weeks	Yes
II.14	1951	M	pterygium	62 years	No	1/year, 1 week	Yes
III.2	1967	M	unaffected	-	No	1/year, 1 week	Yes
III.3	1982	F	unaffected	-	No	1/year, 1 week	Sometimes
III.5	1968	F	pinguecula	38 years	Canada 13 years	1/year, 2 weeks	Sometimes
III.6	1970	F	pinguecula	40 years	Canada 13 years	1/year, 2 weeks	Sometimes

796

797 **Table 1.** Questionnaire results. The Northern Irish family members participating in the study are  
798 listed with age of pterygium diagnosis, averaged 48 years old. The family were shown not to have  
799 had excessive exposure to the sun, spending time in sunny climates from 1 to 3 weeks per years with  
800 most stating that they wear sunglasses.

801

Gene Symbol	Chr	Position	Gene Region	Protein Variant	Conservation to:	Gene expressed in:	Associated Diseases
CTRC	1	15764869	Promoter		Human	pancreas, liver, spleen	chronic pancreatitis
KIF21B	1	200978030	Exonic	p.T105M	Elephant	eye (cornea), brain, blood	multiple sclerosis, inflammatory bowel disease
GALNT14	2	31348076	Intronic; Exonic; 5'UTR	p.A58V	Human	kidney, PNS, blood, eye	breast cancer
CRIMI	2	36706700	Exonic	p.H412P	Lamprey	placenta, kidney, PNS, eye	syndactyly, neuronitis, bovine ocular carcinoma
SPTBN1	2	54886366	Exonic	p.T2107A; p.T2094A	Dog	tongue, soft tissue, eye	fissured tongue, and buphthalmos
VRK2	2	58313555	Exonic; ncRNA	p.G113E; p.G90E	Elephant	lymph node, testis, colon	vaccinia, and hypoxia
PLEK	2	68615546	Exonic	p.N229H	X_tropicalis	bone marrow, lymph node, eye	aarskog-scott syndrome, centronuclear myopathy
HNMT	2	138772049	3'UTR		Dog	bladder, kidney, liver, eye	asthma, eosinophilia-myalgia syndrome
ITGB6	2	160994200	Exonic	p.H469N	Mouse	pancreas, tongue, stomach, eye	bullous keratopathy, mouth disease
WDR12	2	203760891	Exonic	p.L169S	Zebrafish	blood, intestine, thymus, eye	gigantism, and neuronal ceroid lipofuscinosis
CLDN16	3	190106072	Exonic	p.A56fs*16	Rhesus	kidney, ovary, uterus	nephrocalcinosis, and hypomagnesemia primary
CLDN16	3	190106074	Exonic	p.A56P	Rhesus	kidney, ovary, uterus	nephrocalcinosis, and hypomagnesemia primary
PRIM2	6	57398226	Exonic	p.G310V	Human	thymus, ovary, mammary, eye	
STK31	7	23811795	Exonic; ncRNA	p.N598_S600delinsKKI	Elephant	testis	
TYW1	7	66660242	Exonic	p.H632R	Human	stomach, heart, colon, eye	fanconi's anemia, and cytochrome p450
GLDC	9	6556206	Exonic	p.I717V	Zebrafish	small intestine, kidney, liver, eye	glycine encephalopathy
IFNA5	9	21304891	Exonic	p.C122S	Elephant	_____	hemorrhagic fever
DMRTA1	9	22451120	Exonic	p.K242R	Rhesus	_____	metabolic acidosis, prostatitis
MIR4289	9	91360776	microRNA		Dog	_____	
HBG2	11	5276282	Promoter		Rhesus	spleen, thymus, liver	sulfhemoglobinemia, cavernous hemangioma
OR4C16	11	55339462	Promoter		Human	_____	neuronitis
OR10AG1	11	55735808	Exonic	p.H43D	Rhesus	_____	neuronitis
C12orf56	12	64712620	Exonic; Intronic	p.T210I	Human	_____	
ATP8B4	15	50223420	Exonic; ncRNA	p.R513Q	Lamprey	blood, bone marrow	intrahepatic cholestasis
DMXL2	15	51773329	Exonic	p.D1356N; p.D1992N	Chicken	blood, testis, heart, eye	gynecomastia, and myocardial infarction
TPSD1	16	1306802	Exonic	p.I87V	Human	_____	asthma, allergic and inflammatory disorders
TPSD1	16	1306817	Exonic	p.A92T	Human	_____	asthma, allergic and inflammatory disorders
NOMO1	16	18544469	Exonic	p.R418H	Chicken	intestine, cervix, colon, eye	pseudoxanthoma elasticum (PXE)
SRCAP	16	30731568	Exonic	p.R968H	Chicken	Larynx, thymus, spleen, eye (cornea)	floating-harbor syndrome, Eosinophilic angiocentric fibrosis
ZNF319	16	58031913	Exonic	p.A86V	Elephant	bladder, blood, tongue	
PDPR	16	70154480	Exonic	p.T29A	Human	heart, bone, Eye	sarcosinemia
ZFH3	16	72832474	Exonic	p.454_455insH; p.1368_1369insH	X_tropicalis	_____	prostate cancer, acute myocardial infarction
WVOX	16	78466409	Exonic	p.L272F	Rhesus	mammary, tongue, PNS, eye	toxic pneumonitis, and aspiration pneumonitis
GSDMA	17	38122680	Exonic	p.V128L	Human	_____	atopy, gastric cancer
DNAI2	17	72306188	Intronic; Exonic	p.V460V	Elephant	testis, uterus, lung, brain	ciliary dyskinesia
MAN2B1	19	12774537	Exonic	p.P248L	Dog	bone, PNS, intestine, eye	alpha-mannosidosis, alpha-mannosidosis, adult form
CYP4F2	19	15989730	Exonic	p.T472A	Mouse	intestine, blood, liver, muscle	warfarin sensitivity, cytochrome p450
PRKD2	19	47204207	Exonic	p.V167M; p.V324M	Lamprey	Spleen, tongue, ovary, eye	polycystic kidney disease, gastric cancer
NLRP7	19	55451405	Exonic	p.P261Q	X_tropicalis	_____	gestational trophoblastic neoplasm, hydatidiform mole
TTC3	21	38569884	Exonic	p.G1865S	X_tropicalis	Tongue, uterus, heart, eye	down syndrome critical region, down syndrome

Gene Symbol	Translation Impact	SIFT Function Prediction	SIFT Score	PolyPhen-2 Function Prediction	dbSNP ID	NHLBI ESP European Frequency (%)	Co-segregation within the family (IGV)					
							II.2	II.4	III.5	III.6	II.14	II.9
CTRC					144717165		Het	Het	Hom WT	Het	Het	Hom WT
KIF21B	missense	Damaging	0,01	Benign	150551633	0,01	Het	Het	Het	Het	Het	Hom WT
GALNT14	missense	Tolerated	0,26	Benign	41280621		Het	Het	Het	Het	Het	Hom WT
CRIM1	missense	Damaging	0,01	Probably Damaging	113372122	0,29	Het	Het	Het	Het	Het	Hom WT
SPTBN1	missense	Tolerated	0,72	Benign	147989241	0,16	Het	Het	Het	Hom WT	Het	Hom WT
VRK2	missense	Damaging	0,02	Benign	147530902	0,36	Het	Het	Het	Hom WT	Het	Hom WT
PLEK	missense	Tolerated	0,52	Benign	34338164	1,14	Het	Het	Het	Hom WT	Het	Hom WT
HNMT							Het	Het	Het	Hom WT	Het	Hom WT
ITGB6	missense	Tolerated	0,65	Benign	142197545	0,29	Het	Het	Hom WT	Het	Het	Hom WT
WDR12	missense	Damaging	0	Possibly Damaging		0	Het	Het	Hom WT	Het	Het	Hom WT
CLDN16	frameshift				56086318	0	Het	Het	Het	Het	Het	Hom WT
CLDN16	missense	Damaging	0,04	Benign	3214506	0	Het	Het	Het	Het	Het	Hom WT
PRIM2	missense				77436138		Het	Het	Het	Het	Het	Hom WT
STK31	in-frame					0	Het	Het	Het	Het	Het	Hom WT
TYW1	in-frame	Tolerated	0,5	Benign		0	Het	Het	Het	Het	Het	Hom WT
GLDC	missense	Tolerated	1	Benign	117460214	0,03	Het	Het	Hom WT	Het	Het	Hom WT
IFNA5	missense	Damaging	0	Probably Damaging	140371188	0,59	Het	Het	Hom WT	Hom WT	Het	Hom WT
DMRTA1	missense	Tolerated	0,54	Benign	145718826	0,38	Het	Het	Hom WT	Hom WT	Het	Hom WT
MIR4289							Het	Het	Het	Hom WT	Het	Hom WT
HBG2					113622787		Het	Het	Het	Hom WT	Het	Hom WT
OR4C16							Het	Het	Hom m	Het	Het	Hom WT
OR10AG1	in-frame	Activating	1	Benign		0	Het	Het	Hom m	Het	Het	Hom WT
C12orf56	missense	Tolerated	0,13		367932023	0,03	Het	Het	Hom WT	Het	Het	Hom WT
ATP8B4	missense	Damaging	0	Probably Damaging		0	Het	Het	Hom WT	Het	Het	Hom WT
DMXL2	missense	Tolerated	0,17	Benign	144241909	0,02	Het	Het	Hom WT	Het	Het	Hom WT
TPSD1	missense	Tolerated	0,31	Benign	2401930	0	Het	Het	Het	Het	Het	0
TPSD1	missense	Tolerated	0,75	Benign	3993983	0	Het	Het	Het	Het	Het	0
NOMO1	missense	Tolerated	0,23	Benign	140359200	0,12	Het	Het	Hom WT	Hom WT	Het	Hom WT
SRCAP	missense	Damaging	0	Possibly Damaging	368876335	0,01	Het	Het	Hom WT	Het	Het	Hom WT
ZNF319	missense	Tolerated	0,08	Benign		0	Het	Het	Hom WT	Het	Het	0
P DPR	missense	Tolerated	1	Benign	200469748	0	Het	Het	Het	Het	Het	Het
ZFH X3	in-frame					0	Het	Het	Hom WT	Het	Het	Hom WT
W WOX	missense	Tolerated	0,7	Benign	186745328	0,46	Het	Het	Hom WT	Het	Het	Hom WT
GSDMA	missense	Activating	1	Benign		0	Het	Het	Het	Het	Het	Hom WT
DNAI2	synonymous				148947094	0,05	Het	Het	Hom WT	Het	Het	Hom WT
MAN2B1	missense	Damaging	0,01	Possibly Damaging	117843968	0,42	Het	Het	Het	Het	Het	Hom WT
CYP4F2	missense	Tolerated	0,39	Benign	4020346	0	Het	Het	Hom WT	Hom WT	Het	Hom WT
PRKD2	missense	Tolerated	0,15	Benign	45455991	0,98	Het	Het	Hom WT	Het	Het	Hom WT
NLRP7	missense	Damaging	0	Probably Damaging		0	Het	Het	Het	Hom WT	Het	Hom WT
TTC3	missense	Tolerated	0,55	Possibly Damaging		0	Het	Het	Het	Hom WT	Het	Hom WT

804 **Table 2.** Candidate genes analysis. The table shows the 40 genes obtained from Ingenuity and the  
 805 analysis done for each of the associated variants. The selection was mainly based on the expression  
 806 of the gene in the eye (TIGER database), the SIFT and Polyphen predictions and known diseases  
 807 association (literature review). Subsequently the aminoacid conservation through vertebrate species  
 808 (BLAST analysis) and co-segregation of the mutation in pterygium (II.2, II.4, II.14) and pinguecula  
 809 (III.5, III.6) affected members and not in the II.9 unaffected family member (IGV analysis) was  
 810 considered. This screening led to the selection of *CRIMI* as the best candidate considering those  
 811 parameters.

812

813 A.

Patient NI	Year of birth	Sex	Eye condition
1	1942	M	pterygium
2	1950	M	minor pterygium
3	1931	M	pterygium
4	1954	M	pterygium
5	1946	M	pterygium
6	1975	M	pterygium
7	1941	M	pterygium
8	1958	F	pterygium
9	1984	F	pterygium
10	1934	M	pterygium
11	1974	F	pterygium
12*	1967	F	pterygium family

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818 B.

Patient Bolivia	Year of birth	Sex	Eye condition
B1	1965	F	pterygium
B2		M	pterygium
B3	1949	M	pterygium
B4	1957	M	pterygium
B5	1999	M	pterygium
B6	1952	M	pterygium
B7	1947	F	pterygium
B8	1941	M	pterygium
B9	1990	M	pterygium

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820 **Table 3.** Individual pterygium participants. **(A).** A list of pterygium affected individuals collected in  
821 the UK and the relevant information obtained through the questionnaire. \*Patient 12 denotes the  
822 pterygium affected II.1 family member previously studied<sup>27</sup>. **(B).** List of pterygium affected  
823 individuals collected in Bolivia and the year of birth and sex of participants.

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<b>CRIM1 sequence</b>	<b>Primer sequence (5' to 3')</b>
T7_F	TAATACGACTCACTATAGGG
Seq1_R	GCAGAATGTGCAGTCGTCTT
Seq1_F	TGATCGAGGGTTATGCTCCT
Seq2_F	TACTACGTGCCCCAAGGAGA
Seq2_R	GGCACTTTCACAGGGTTTGT
Seq3_F	TGCCGGGAATGCTACTGT
Seq3_R	ACAGAAGGGCAGGACTCAGA
Seq4_F	CTGAGTCCTGGAAGCCTGAC
Seq4_R	CCTGGAGGTGACCCATATCT
Seq5_F	AACCATCGAGGAGAGGTTGA
Seq5_R	TCGTCTTCCGTCTTTTGAAAC

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835 **Supplementary Table 1.** Primers used to validate that the whole of the CRIM1 sequence was inserted  
836 into the pcDNA3.1 plasmid and to check the presence of the H412P mutation introduced by site  
837 directed mutagenesis.

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<b>CRIM1 VWFs</b>	<b>Primer sequence (5' to 3')</b>
Exon6_F (VWF1)	TTGAAAAACATCAAAGGACACAA
Exon6_R (VWF1)	CCATGTATGCTCCTGTTAATCTG
Exon7_F (VWF2)	GATGACTAGAACCCAGGGAAAA
Exon7_R (VWF2)	AGCAGACATTATGCCCAAGG
Exon11_F (VWF3)	GCCTGTTTCTCCTGTGCAGT
Exon11_R (VWF3)	TGCAAGGCAGAAGTCATTTG
Exon12_F (VWF4)	CCAGGCTTTCAAGAGTTGGA
Exon12_R (VWF4)	GGGTCCCACAGAATGACAAC
Exon13_F (VWF5)	CTGGCCAACAGCATCTTCTT
Exon13_R (VWF5)	GACATGTCAAGCAGGGAAAAA
Exon14_F (VWF6)	AAGATCGTGTGCGTTGTCAC
Exon14_R (VWF6)	GTCGAGCTCTGCTTCGATTT

840

841 **Supplementary Table 2.** Primers used to verify the presence of other mutations in all the VWF  
842 domains of CRIM1.

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