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Stickler syndrome caused by *COL2A1* mutations: genotype-phenotype correlation in a series of 100 patients

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ABSTRACT

Stickler syndrome is an autosomal dominant connective tissue disorder caused by mutations in different collagen genes. The aim of our study was to define more precisely the phenotype and genotype of Stickler syndrome type 1 by investigating a large series of patients with a heterozygous mutation in COL2A1. In 188 probands with the clinical diagnosis of Stickler syndrome, the COL2A1 gene was analysed by either a mutation scanning technique or bidirectional fluorescent DNA sequencing. The effect of splice site alterations was investigated by analysing mRNA. MLPA analysis was used for the detection of intragenic deletions. We identified 77 different COL2A1 mutations in 100 affected individuals. Analysis of the splice site mutations revealed unusual RNA isoforms, most of which contained a premature stop codon. Vitreous anomalies and retinal detachments were found more frequently in patients with a COL2A1 mutation compared to the mutation negative group ($p<0.01$). 20 of the 23 sporadic patients with a COL2A1 mutation had either a cleft palate or retinal detachment with vitreous anomalies. The presence of vitreous anomalies, retinal tears or detachments, cleft palate and a positive family history were shown to be good indicators for a COL2A1 defect. In conclusion, we confirm that Stickler syndrome type 1 is predominantly caused by loss-of-function mutations in the COL2A1 gene since more than 90% of the mutations were predicted to result in nonsense mediated decay. Based on binary regression analysis we developed a scoring system that may be useful when evaluating patients with Stickler syndrome.

INTRODUCTION

Stickler syndrome (MIM# 108300) is a connective tissue disorder first described by Stickler et al in 1965. It is characterized by ocular, orofacial, auditory and skeletal manifestations with considerable intra- and interfamilial variability.[1, 2] The incidence is estimated to range between 1 in 7,500 to 1 in 9,000 newborns (http://ghr.nlm.nih.gov/condition%3Dsticklersyndrome US Dept of Health). The most characteristic ocular features are congenital myopia, vitreous alterations, cataract, glaucoma and a high risk of spontaneous retinal detachments. The orofacial changes include cleft palate, midfacial hypoplasia, low nasal bridge and micrognathia. Joint pain is common in childhood and osteoarthrosis may be apparent from the third or fourth decade. Radiographs may show signs of a spondyloepiphyseal dysplasia. Mild sensorineural hearing loss, mainly for the high tones, can be present in Stickler syndrome type 1 (*COL2A1* gene), more severe sensorineural hearing loss is usually found in the other types of Stickler syndrome.[3, 4]

At present, at least 3 types of autosomal dominant Stickler syndrome have been discerned. A correlation between these different types and their accompanying vitreous anomalies has been suggested.[5] ‘Membranous’ or type 1 vitreous has been associated with Stickler syndrome type 1 caused by heterozygous mutations in the *COL2A1* gene (MIM# 108300).[6] Type 2 or ‘beaded’ vitreous is mainly found in patients with Stickler syndrome type 2 which is due to a heterozygous mutation in the *COL11A1* gene (MIM# 604841).[3, 7] Stickler syndrome type 3 or ‘non-ocular Stickler syndrome’ refers to the phenotype of patients with a mutation in the *COL11A2* gene that is not expressed in the eye (MIM# 184840).[8] In addition to the different types of autosomal

136 dominant Stickler syndrome, recently also a recessive form of Stickler syndrome,
137 caused by a mutation in the *COL9A1* gene, has been described (MIM# 120210).[9]
138 Stickler syndrome type 1 is the most common form. The majority of *COL2A1*
139 mutations identified in patients with Stickler syndrome type 1 are predicted to result in
140 nonsense mediated decay (NMD). On the other hand, missense mutations (usually
141 glycine substitutions) in *COL2A1* usually result in short stature disorders such as
142 achondrogenesis type II/hypochondrogenesis, spondyloepiphyseal dysplasia (SEDC),
143 Kniest dysplasia, spondyloperipheral dysplasia (SPD) and Torrance dysplasia (MIM#
144 200610, 183900, 156550, 271700, 151210).[5, 10, 11]
145 The aim of this study was to define more precisely the phenotype and genotype
146 of Stickler syndrome type 1 by investigating a large series of Stickler syndrome patients
147 with a heterozygous mutation in the *COL2A1* gene.
148

MATERIALS & METHODS

Evaluation of phenotype

Over the past 10 years, blood or DNA samples from 278 individuals were referred for mutation analysis of the *COL2A1* gene in order to confirm or exclude the clinical diagnosis of Stickler syndrome.

Information on clinical and radiographic features of each patient was requested by using a specific questionnaire (Supplementary Table 1). Ninety patients were excluded from the study because insufficient clinical data were available (in 11 of those patients a *COL2A1* mutation was identified). Each patient in the group of 188 remaining subjects had two or more of the following features reminiscent of Stickler syndrome: myopia, spontaneous retinal detachment, cleft palate, sensorineural hearing loss and arthropathy. Informed consent was obtained from each enrolled patient.

Analysis of genomic DNA

Genomic DNA was extracted from blood samples by standard procedures, followed by touchdown PCR amplification of the *COL2A1* gene using forward and reverse primers located in the flanking introns. The PCR products were analysed by gel electrophoresis and visualized by ethidium bromide staining on 2% agarose gels.

Mutation screening was performed by SSCP and CSGE (period 1997-2002) or by DHPLC analysis (period 2003-2006) using the WAVE DNA fragment analysis system (Transgenomic, Cheshire, UK).[12, 13, 14] All fragments showing an aberrant pattern were directly sequenced on the ABI PRISM 3730 automated sequencer (Applied Biosystems, Foster City, CA) using the BigDye terminator cycle sequencing chemistry. From 2007 on, direct sequencing of all 54 exons was performed. These obtained sequences were compared to the wild-type sequence as submitted to GenBank

Accession number NM_001844. The nucleotides were numbered starting from the first base of the start codon (ATG) of the cDNA reference sequence. Amino acid residues were numbered from the first methionine (start codon for translation) of the procollagen α1(II)-chain (GenBank Accession number L10347).

RNA studies

In patients with a splice site mutation, an EBV cell line or skin biopsy was requested for analysis of mRNA splicing. In order to stabilize mutant *COL2A1* mRNA, cycloheximide (Sigma, www.sigmaaldrich.com) was added to the cultures, followed by mRNA isolation and cDNA preparation. Nested PCR was used to obtain sufficient PCR fragments for direct sequencing.

MLPA analysis

Multiplex ligation-dependent amplification (MLPA) was set up, following the directions provided by the manufacturer (MRC Holland, Amsterdam, The Netherlands) (www.MPLA.com).[15] The probe set for COL2A1 (SALSA MLPA kit P214) covering exons 1, 4, 6, 8, 10, 16, 17, 19, 20, 24, 27, 29, 31, 35, 39, 43, 46, 49, 51 and 54 was used.

Binary logistic regression analysis/statistics

The formula for the proposed scoring system was developed using binary (mutation positive or not) logistic regression analysis.[16, 17, 18] The parameters tested in the model comprised: vitreous abnormalities, retinal abnormalities, flat face, micrognathia, retinal tear and/or detachment, cataract, low nasal bridge, cleft palate, positive family history, myopia, conductive hearing loss, premature arthropathy, hypermobility, epiphyseal dysplasia on X-rays and sensorineural hearing loss. The weight (score) for each characteristic in the scoring system was proportional to its regression coefficient in

197 the model. To simplify the scoring system the scores were rounded to positive integers
198 and the scores of the characteristics with lowest significant regression coefficients were
199 conventionally given a score value of one and the intercept of the linear predictor was
200 neglected. Otherwise, no recalibration, shrinkage factor or model revision or extension
201 seemed to be needed to study the whole study population. Calibration of the scoring
202 system was further evaluated with the Hosmer-Lemeshow test. The clinical applicability
203 of the obtained score was evaluated for several thresholds using conventional receiver
204 operating characteristics (positive and negative predictive value, sensitivity and
205 specificity). All statistical analyses were performed with SPSS 15.0 for Windows (SPSS
206 Inc. Chicago, IL).
207

RESULTS

In 100/188 individuals referred with a potential diagnosis of Stickler syndrome, a heterozygous *COL2A1* mutation was identified. This panel of 77 different mutations included 1 deletion of the entire gene [19], 13 nonsense mutations, 21 deletions, 1 insertion, 9 duplications, 2 combinations of an insertion and a deletion, 22 splice site alterations, 1 synonymous mutation, 2 missense mutations resulting in an arginine-to-cysteine substitution [20] and 5 missense mutations substituting a glycine residue in the triple helical domain of the protein. The mutations were distributed over the entire gene and no hot spot regions were apparent (Table 1). Thirteen mutations were observed in more than one proband: c.625C>T, p.Arg209X and c.1833+1G>A, p.GlyfsX619 were found in four patients each; c.3106C>T, p.Arg1036X occurred five times (Table 1). Two mutations were located in the alternatively spliced exon 2. The first one, a duplication of 23 nucleotides (c.211_233dup; p.Glu79ThrfsX2) causes a frameshift that leads to a premature stop codon within the exon itself. The patient with this mutation only had ocular features (retinal detachment) of Stickler syndrome as expected since exon 2 is retained in the eye but spliced out in the cartilage.[21, 22, 23] The second patient had the deletion c.264_276del; p.Cys89SerfsX24 that causes a frameshift with a premature stop codon in exon 3. However, this patient had both ocular and extra-ocular manifestations of Stickler syndrome including vitreal abnormalities, a retinal detachment, flat face, sensorineural hearing loss, arthropathy and epiphyseal changes on radiographs.

In the skin fibroblasts or the EBV cell line available from 13 patients with 12 different splice site alterations, cDNA analysis showed that each splice site alteration resulted in a premature stop codon (data not shown). For the three splice site mutations,

232 multiple isoforms of mRNA were detected. In each case at least one isoform harboured
233 a premature stop codon (Supplementary Figure 1: isoforms A3, B2 and C2). In the
234 additional isoforms A1, B1 and C1 only skipping of the adjacent exon was observed. In
235 the C1 isoform skipping of even three consecutive exons (51-53) had occurred. These
236 exons constitute the carboxypropeptide of the procollagen α1(II)-chain, which is
237 necessary for chain association and initiation of the triple helix formation.[24]
238 Consequently, the resulting truncated protein will most likely be lost and not
239 incorporated into the collagen trimer. In the isoform A2, exon 7 was deleted but intron
240 5 retained, the latter containing an in-frame stop codon. Both patients harbouring the
241 c.430-1G>C and c.4074+1G>T splice site mutation suffered from myopia, vitreoretinal
242 abnormalities and spontaneous retinal detachments. They also showed a flat face. The
243 individual with the c.3003+5G>A splice site mutation was born with a Pierre-Robin
244 anomaly and had myopia, a retinal detachment and cataract. He also suffered from
245 conductive hearing loss. His affected father had a history of spontaneous bilateral retinal
246 detachments in childhood.
247 One patient was heterozygous for a synonymous mutation (c.2862C>T;
248 p.Gly954Gly) in exon 42. Since this mutation was cosegregating with Stickler
249 syndrome in the affected family, the pathogenic effect was further explored at the
250 mRNA level. cDNA analysis showed that this mutation generated a cryptic splice site
251 35 nucleotides upstream of the normal donor splice site in intron 42, resulting in a
252 frameshift with a premature stop codon (Supplementary Figure 2).
253 The nonsense (p.Trp1293X) and frameshift mutations (p.Cys1289ProfsX3,
254 p.Ile1300ThrfsX15, p.Asn1303ThrfsX9) residing in the carboxypropeptide were
255 predicted to result in NMD since they occur before the last 50 nucleotides of the last

256 exon-exon junction (Table 1).[25] In the patient with the splice site alteration in intron
257 53 (c.4317+2T>C), the splice site prediction program
258 (http://www.fruitfly.org/seq_tools/splice.html) computed an insertion of a part of the
259 intron 53 containing an in-frame stop codon.

260 Since only 100 mutations were identified in a series of 188 patients, we decided
261 to expand the molecular analysis with MLPA to explore the possibility of missed
262 intragenic deletions. For this analysis we selected 20 patients in whom we strongly
263 suspected the diagnosis of Stickler syndrome because of the presence of severe myopia,
264 retinal detachment and/or cleft palate. However, no additional mutations were identified
265 in these affected individuals.

266 In a next step we evaluated the clinical and radiographic features in our series of
267 188 patients and looked for differences between the mutation positive (n=100) and
268 mutation negative (n=88) group. The results are summarized in Figure 1. A positive
269 family history, orofacial anomalies (cleft palate, low nasal bridge, flat face,
270 micrognathia) and vitreoretinal changes were more frequently (p value ≤ 0.05) present
271 in the mutation positive group. On the other hand, sensorineural hearing loss was
272 observed more frequently in the mutation negative group ($p<0.005$). 20/23 of the
273 sporadic patients with a *COL2A1* mutation had either a cleft palate or retinal
274 detachment(s) with vitreous anomalies and myopia.

275 To determine the discriminating power of these features, we performed a binary
276 logistic regression analysis. The following characteristics were most distinguishing
277 between both groups : a) vitreous abnormalities, b) retinal abnormalities, c) history of
278 retinal tear and/or detachment, d) low nasal bridge, e) cleft palate, f) micrognathia and
279 g) positive family history (Figure 2). Based on the regression coefficient of each

distinguishing characteristic, a specific scoring system was proposed. The highest score (score 5) was attributed to retinal abnormalities and positive family history, a score of 4 was assigned to cleft palate and vitreous abnormalities, a retinal tear and/or detachment represented a score of 3, whereas low nasal bridge and micrognathia received the lowest score (score 1) (Table 2). When applying this scoring system to each patient, we observed a higher median score for patients with a *COL2A1* mutation compared to those without a mutation (11.5 versus 6). The calculated score ranges from 0 to 21 with a theoretical maximum of 23. The distribution of the score for mutation positive and mutation negative cases is shown in Figure 3. 75% of the patients with a *COL2A1* mutation had a total score ≥ 9 (Figure 4). The presence of vitreoretinal anomalies and a retinal detachment yields a total score of 12, illustrating the importance of a thorough ophthalmological evaluation in patients with Stickler syndrome.

DISCUSSION

The past decade we have identified a large series of *COL2A1* mutations in a group of patients referred with the diagnosis of Stickler syndrome. The availability of these data prompted us to retrospectively analyse both genotype and phenotype of these patients. With this study we aimed to define more precisely the phenotype of Stickler syndrome type 1 and were interested in identifying discriminating features between patients with and those without a *COL2A1* mutation. In addition, we wanted to investigate in what respect Stickler syndrome type 1 mutations were different from other *COL2A1* mutations causing the type II collagenopathies with short stature. More precisely, we wanted to learn if all Stickler syndrome mutations were predicted to have a loss-of-function effect on the procollagen α1(II)-chain.

Sufficient clinical and radiographic data were available on 188 probands and in 100 of these individuals a heterozygous *COL2A1* mutation was identified. The 77 different mutations were distributed over the entire gene and no regions of mutation clustering were found. Thirteen mutations were observed in more than one proband, with 10 involving a CpG dinucleotide. One patient was heterozygous for a deletion of the entire gene and details have been published earlier.[19] The 34 smaller and intragenic deletions, insertions, duplications and insertion-deletions were all out of frame and therefore predicted to result in NMD. A similar effect was demonstrated for the synonymous mutation (p.Gly954Gly) which created a cryptic splice site (Supplementary Figure 2). This mutation is the second example of an apparently silent *COL2A1* mutation that alters RNA splicing, illustrating the importance of studying the effect of so-called synonymous mutations at the mRNA level.[26] Analysis of cDNA also allowed us to study the effect of 12 different splice site alterations. In addition, it

317 gave us more insights into the complexity of mRNA splicing of the *COL2A1* gene. Each
318 splice site mutation was shown to create at least one isoform with a frameshift and
319 premature stop codon as a consequence (Supplementary Figure 1). In addition, some
320 unexpected splice site outcomes were observed with skipping of one or more
321 consecutive exons and even retention of introns more remote from the mutation. As
322 shown before for collagen types I and V, introns are not consecutively removed in a 5'
323 to 3' direction which may explain some unusual RNA isoforms observed in our
324 patients.[27, 28]

325 In addition to the above mentioned hypomorphic mutations, also 7 different
326 missense mutations were identified in this series of patients. Five mutations
327 (p.Gly216Asp; p.Gly219Arg; p.Gly222Val; p.Gly492Asp; p.Gly1131Ala) were
328 predicted to result in a glycine substitution. Glycine substitutions in the triple helical
329 domain usually have a dramatic effect by hampering proper triple helix formation of the
330 collagen trimer. They usually result in a type II collagen disorder with either lethal
331 outcome (achondrogenesis type 2/hypochondrogenesis) or severe short stature (SEDC,
332 Kniest dysplasia). Upon review of the literature and our own data, glycine substitutions
333 causing these short stature phenotypes never seem to occur amino-terminal to the
334 glycine residue at position 303.[29] Glycine substitutions upstream of this residue seem
335 to have a less deleterious effect on collagen trimer formation and function which may
336 explain the Stickler syndrome phenotype in our patients with the p.Gly216Asp,
337 p.Gly219Arg or p.Gly222Val substitution. For the more carboxy-terminally located
338 missense mutations, there is a less clear correlation between the location of the glycine
339 substitution and the phenotypic outcome. The nature of the substituting amino acid may
340 also play a role as is exemplified by the p.Gly492Val mutation that causes

341 spondyloepiphyseal dysplasia [30] and the Gly492Asp mutation that results in Stickler
342 syndrome (our series).
343 Not only missense mutations substituting a glycine residue were identified but
344 also two different missense mutations changing an arginine for a cysteine residue
345 (Arg565Cys; Arg904Cys) were found in a group of 5 patients. These substitutions
346 involve an arginine residue in the X position of the Gly-X-Y triplet.[20, 31] As we
347 reported before, substituting an arginine in the X position seems to cause Stickler
348 syndrome, whereas substituting an arginine in the Y position rather causes a type II
349 collagenopathy without ocular involvement.[20] Cysteine residues are normally not
350 present in the triple helical domain of the procollagen α1(II)-chain.[24] The insertion of
351 such a residue may generate aberrant disulphide bonds between mutant procollagen
352 chains and as such hamper proper chain alignation and trimer formation. In these
353 circumstances, the mutation may have a loss-of-function effect on the protein.
354 The second major goal of this study was to delineate the phenotype of Stickler
355 syndrome type 1 and to try and identify distinguishing characteristics between patients
356 with and without a *COL2A1* mutation. In the group of 100 patients with a mutation,
357 89% had myopia and 55% suffered from at least one episode of spontaneous retinal
358 detachment. Vitreous abnormalities were identified in 42% of the affected individuals,
359 but it proved difficult for most referring ophthalmologists to classify these anomalies
360 into either a type 1 or type 2 vitreous anomaly. Sixty per cent of the mutation positive
361 patients presented with a cleft palate at birth. Binary logistic regression analysis
362 revealed that the ocular and orofacial features were the most distinguishing clinical
363 characteristics between both groups. An affected first degree relative, the presence of
364 vitreoretinal anomalies and cleft palate were good indicators for Stickler syndrome type

365 1. Their presence in a patient with Stickler syndrome increases the likelihood of finding
366 a *COL2A1* mutation upon molecular analysis. On the other hand, severe sensorineural
367 hearing loss was more frequently observed in the mutation negative group (Figure 1).
368 The latter confirms the findings of previous studies indicating that hearing loss is more
369 prevalent and pronounced in type 2 Stickler syndrome.[3] Some features (e.g. myopia)
370 were not included in the scoring system because they were frequently reported in both
371 groups and thus only had a weak discriminating power. Interestingly, there was no
372 statistical difference in the occurrence of early-onset osteoarthrosis and
373 spondyloepiphyseal anomalies between the group with and without a *COL2A1*
374 mutation. When applying the proposed score system, a higher total score was found in
375 the group of patients with a *COL2A1* mutation (Figures 3 and 4), which is in contrast to
376 previous studies in which no differences were observed.[32] Nevertheless, a
377 considerable overlap between both groups was present. This overlap is most likely due
378 to an age-of-onset effect in the mutation positive group and genetic heterogeneity in the
379 mutation negative group. In the latter group, individuals with a *COL11A1* mutation may
380 be present (especially those with severe hearing loss) as well as patients with an
381 undetected *COL2A1* mutation (false negative patients). Indeed, samples referred at the
382 beginning of the study were analysed with less sensitive mutation screening techniques
383 such as SSCP and CSGE. In addition, deletions involving one particular amplicon will
384 be missed by sequencing analysis. However, MLPA analysis in a selected group of
385 patients failed to unravel new mutations. Also, regions outside the coding sequences
386 such as the promoter were not analysed in this study. Lower scores in the mutation
387 positive group may be due to the young age of the affected inviduals not yet showing all

388 features (such as retinal detachments) of Stickler syndrome type 1. Of the 16 cases with
389 a score of ≤ 8, fourteen patients were less than 14 years of age (Table 1).
390 In conclusion, this study conducted in a large series of patients, confirms that
391 Stickler syndrome type 1 is predominantly caused by loss-of-function mutations in the
392 *COL2A1* gene. Only 10% of the gene alterations were missense mutations residing in
393 the triple helical domain, some of which may still exert a hypomorphic effect (e.g. the
394 arginine-to-cysteine substitutions). Vitreoretinal abnormalities including the occurrence
395 of a retinal tear or detachment were statistically more frequent in Stickler syndrome
396 patients with a *COL2A1* mutation compared to those without a mutation. Together with
397 cleft palate and a positive family history these characteristics were shown to be good
398 indicators for a type II collagen defect (in contrast to severe sensorineural hearing loss).
399 Finally, binary regression analysis allowed us to develop a scoring system that
400 highlighted the importance of a thorough vitreoretinal assessment when evaluating
401 individuals suspected with Stickler syndrome type 1.

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Supplementary information is available at the European Journal of Human Genetics website (http://www.nature.com/ejhg/index.html)

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Table 1: Summary of 77 different *COL2A1* mutations identified in a series of 100 affected individuals

Patient ID	Age	Score	Exon/Intron	cDNA	Protein	Mutation type	Mutation effect
1	46	15		del COL2A1	del COL2A1	large deletion	deletion[19]
2	54	13	02	c.211_233dup	p.Glu79ThrfsX2	duplication	frameshift
3	58	17	02	c.264_276del	p.Cys89SerfsX24	deletion	frameshift
4	4	6	IVS 04	c.342+1G>A	p.Asp114_Ile115insIleSerAlaAsnTyr-SerHisProValLeuGlnLeuLeuX14	RNA processing	insertion with premature stop codon
5	42	17	IVS 06	c.430-1G>C	p.Gly144ValfsX54; p.Gln125_Gly126insArgGluGlyGlu-AsnLeuPheLeuArgProPheLeuAlaAla-GlnValThrAspLeuX20; p.Lys143_Asn178delExon7**	RNA processing	frameshift; insertion with premature stop codon; exon deletion
6	6	6	07	c.492delT	p.Gly165ValfsX34	deletion	frameshift
7	3	*6*	*09*	*c.625C>T*	*p.Arg209X*	*nonsense*	*premature stop codon*
8	6	*11*	*09*	*c.625C>T*	*p.Arg209X*	*nonsense*	*premature stop codon*
9	19	*13*	*09*	*c.625C>T*	*p.Arg209X*	*nonsense*	*premature stop codon*
10	12	*14*	*09*	*c.625C>T*	*p.Arg209X*	*nonsense*	*premature stop codon*
11	22	15	09	c.647G>A	p.Gly216Asp	missense	glycine substitution
12	8	8	IVS 09	c.654+1G>A	ND	RNA processing	ND
13	34	10	10	c.655G>C	p.Gly219Arg	missense	glycine substitution
14	27	14	10	c.665G>T	p.Gly222Val	missense	glycine substitution
15	24	11	11	c.744delT	p.Gly249GlufsX59	deletion	frameshift
16	45	18	12	c.793delG	p.Glu265fsX43	deletion	frameshift
17	37	14	IVS 13	c.870+5 G> A	ND	RNA processing	ND
18	30	9	IVS 14	c.925-1G>A	p.Lys308_Gly309insGluPheAlaGly-GlyGlnGluTrpGlyProArgHisX13	RNA processing	insertion with premature stop codon
19	67	*12*	*17*	*c.1030C>T*	*p.Arg344X*	*nonsense*	*premature stop codon*
20	9	*9*	*17*	*c.1030C>T*	*p.Arg344X*	*nonsense*	*premature stop codon*
21	62	21	IVS 18	c.1123-1G>A	p.Gly375ValfsX253	RNA processing	frameshift
22	6	7	19	c.1172delC	p.Pro391LeufsX238	deletion	frameshift
23	11	11	IVS 19	c.1221+1G>A	ND	RNA processing	ND
24	43	12	21	c.1311_1313delinsCA	p.Gly438ThrfsX191	deletion/insertion	frameshift
25	33	12	23	c.1428_1429insTGGC	p.Gly477TrpfsX12	insertion	frameshift
26	13	8	23	c.1475G>A	p.Gly492Asp	missense	glycine substitution
27	40	*13*	*25*	*c.1597C>T*	*p.Arg533X*	*nonsense*	*premature stop codon*
28	10	*10*	*25*	*c.1597C>T*	*p.Arg533X*	*nonsense*	*premature stop codon*
29	12	15	IVS 25	c.1680+2delGTinsAA	ND	RNA processing	ND
30	24	*10*	*26*	*c.1693C>T*	*p.Arg565Cys*	*missense*	*arginine-to-cysteine substitution[20]*
31	20	*8*	*26*	*c.1693C>T*	*p.Arg565Cys*	*missense*	*arginine-to-cysteine substitution[20]*
32	9	*7*	*26*	*c.1693C>T*	*p.Arg565Cys*	*missense*	*arginine-to-cysteine substitution[20]*
33	11	19	27	c.1777C>T	p.Gln593X	nonsense	premature stop codon
34	14	14	27	c.1828delG	p.Ala610ProfsX19	deletion	frameshift
35	11	*10*	*IVS 27*	*c.1833+1G>A*	*ND*	*RNA processing*	*ND*

Patient ID	Age	Score	Exon/Intron	cDNA	Protein	Mutation type	Mutation effect
36	36	*12*	*IVS 27*	*c.1833+1G>A*	*p.Gly609GlyfsX1*	*RNA processing*	*frameshift*
37	40	*14*	*IVS 27*	*c.1833+1G>A*	*ND*	*RNA processing*	*ND*
38	17	*17*	*IVS 27*	*c.1833+1 G>A*	*p.Gly609GlyfsX1*	*RNA processing*	*frameshift*
39	14	10	IVS 28	c.1888-2A>G	p.Gly630MetfsX53	RNA processing	frameshift
40	13	6	29	c.1931delC	p.Pro644LeufsX144	deletion	frameshift
41	40	*19*	*30*	*c.1957C>T*	*p.Arg653X*	*nonsense*	*premature stop codon*
42	41	*12*	*30*	*c.1957C>T*	*p.Arg653X*	*nonsense*	*premature stop codon*
43	4	6	IVS 32	c.2094+1G>A	ND	RNA processing	ND
44	35	9	IVS 32	c.2095-1G>A	ND	RNA processing	ND
45	40	*8*	*33*	*c.2101C>T*	*p.Arg701X*	*nonsense*	*premature stop codon*
46	31	*10*	*33*	*c.2101C>T*	*p.Arg701X*	*nonsense*	*premature stop codon*
47	8	11	IVS 33	c.2193+2T>C	ND	RNA processing	ND
48	43	14	34	c.2257_2264delGGCGAGAG	p.Glu754SerfsX13	deletion	frameshift
49	5	5	34	c.2263_2264delAG	p.Arg755GlyfsX14	deletion	frameshift
50	9	*11*	*35*	*c.2353C>T*	*p.Arg785X*	*nonsense*	*premature stop codon*
51	14	*10*	*35*	*c.2353C>T*	*p.Arg785X*	*nonsense*	*premature stop codon*
52	37	*7*	*35*	*c.2353C>T*	*p.Arg785X*	*nonsense*	*premature stop codon*
53	33	*8*	*IVS 35*	*c.2355+5G>A*	*ND*	*RNA processing*	*ND*
54	38	*13*	*IVS 35*	*c2355+5G>A*	*p.Arg785_Gly786insValAsnGluCys-GlyLeuLeuAspCysTrpAlaPheGlySerX15*	*RNA processing*	*insertion with premature stop codon*
55	11	11	36	c.2381dupC	p.Gly795TrpfsX6	duplication	frameshift
56	5	*10*	*36*	*c.2382delT*	*p.Gly795Alafs86*	*deletion*	*frameshift*
57	41	*12*	*36*	*c.2382delT*	*p.Gly795Alafs86*	*deletion*	*frameshift*
58	14	9	38	c.2467G>T	p.Glu823X	nonsense	premature stop codon
59	44	9	38	c.2493dupA	p.Pro832ThrfsX11	duplication	frameshift
60	66	13	IVS 38	c.2517+2T>G	ND	RNA processing	ND
61	24	15	IVS 38	c.2518-1 G>A	p.Gly840ValfsX41	RNA processing	frameshift
62	41	17	39	c.2588-2604delCTGG TCCTCAGGGCCCC	p.Pro863LeufsX16	deletion	frameshift
63	39	17	40	c.2659C>T	p.Arg887X	nonsense	premature stop codon
64	12	12	40	c.2673dupC	p.Ala895SerfsX49	duplication	frameshift
65	33	9	40	c.2673delC	p.Pro893ArgfsX135	deletion	frameshift
66	9	*14*	*41*	*c.2710C>T*	*p.Arg904Cys*	*missense*	*arginine-to-cysteine substitution[20]*
67	18 (8 at exam)	*0*	*41*	*c.2710C>T*	*p.Arg904Cys*	*missense*	*arginine-to-cysteine substitution[20]*
68	70	9	41	c.2715dupT	p.Gly906TrpfsX38	duplication	frameshift
69	40	17	41	c.2719dupC	p.Gly909ArgfsX35	duplication	frameshift
70	44	9	42	c.2813delC	p.Pro938LeufsX90	deletion	frameshift
71	58	9	42	c.2839C>T	p.Gln947X	nonsense	premature stop codon
72	12	10	42	c.2862C>T	p.Gly954Gly*	synonymous	frameshift
73	11	8	IVS 43	c.3003+1G>A	ND	RNA processing	ND
74	20	13	IVS 43	c.3003+5G>A	p.Gly966_Ser1001del; p.Gly990GlyfsX1**	RNA processing	deletion; frameshift

Patient ID	Age	Score	Exon/Intron	cDNA	Protein	Mutation type	Mutation effect
75	32	16	44	c.3081_3087delGACGGT insCCTGG	p.Thr1028LeufsX100	deletion/insertion	frameshift
76	18	*17*	*44*	*c.3106C>T*	*p.Arg1036X*	*nonsense*	*premature stop codon*
77	39	*14*	*44*	*c.3106C>T*	*p.Arg1036X*	*nonsense*	*premature stop codon*
78	10	*11*	*44*	*c.3106C>T*	*p.Arg1036X*	*nonsense*	*premature stop codon*
79	47	*13*	*44*	*c.3106C>T*	*p.Arg1036X*	*nonsense*	*premature stop codon*
80	45	*10*	*44*	*c.3106C>T*	*p.Arg1036X*	*nonsense*	*premature stop codon*
81	49	13	IVS 44	c.3111+1G>T	p.Glu1033LysfsX4	RNA processing	frameshift
82	8	11	IVS 44	c.3112-1G>A	p.Gly1038GlufsX92	RNA processing	frameshift
83	29	12	45	c.3137delC	p.Pro1046LeufsX84	deletion	frameshift
84	8	12	45	c.3137dupC	p.Gly1047TrpfsX11	duplication	frameshift
85	17	13	45	c.3138delT	p.Gly1047AlafsX83	deletion	frameshift
86	20	12	46	c.3228delT	p.Gly1077AlafsX53	deletion	frameshift
87	35	18	46	c.3258_3261delAGAC	p.Asp1087GlufsX42	deletion	frameshift
88	42	9	47	c.3325delC	p.Gln1109ArgfsX21	deletion	frameshift
89	18	11	48	c.3392G>C	p.Gly1131Ala	missense	glycine substitution
90	8	*11*	*50*	*c.3574C>T*	*p.Arg1192X*	*nonsense*	*premature stop codon*
91	47	*16*	*50*	*c.3574C>T*	*p.Arg1192X*	*nonsense*	*premature stop codon*
92	40	*18*	*50*	*c.3574C>T*	*p.Arg1192X*	*nonsense*	*premature stop codon*
93	42	11	51	c.3623delC	p.Pro1208LeufsX19	deletion	frameshift
94	33	12	51	c.3641dupC	p.Gly1215TrpfsX38	duplication	frameshift
95	41	10	51	c.3864-3865delCT	p.Cys1289ProfsX3	deletion	frameshift
96	11	10	51	c.3878G>A	p.Trp1293X	nonsense	premature stop codon
97	55	12	52	c.3891_3898dupCTACTGGA	p.Ile1300ThrfsX15	duplication	frameshift
98	53	12	52	c.3903delC	p.Asn1303ThrfsX9	deletion	frameshift
99	52	17	IVS 52	c.4074+1 G>T	p.Gln1238_Leu1411del; p.Trp1348CysfsX17**	RNA processing	deletion; frameshift
100	8	10	IVS 53	c.4317+2T>C	ND	RNA processing	ND

indication N-propeptide (p.26-181) - triple helical domain (p.201-1214) - C-propeptide (p.1242-1487)

IVS: intervening sequence

ND: not determined

Items in italic are recurrent mutations

Exons are numbered 1-54

cDNA mutations are numbered starting from the first base of the

Amino acid mutations were numbered from the first

* Synonymous mutation: see Supplementary Figure 2

** Splice site mutations with multiple isoforms: see Supplementary Figure 1

Score as calculated by the proposed scoring system in Table 2

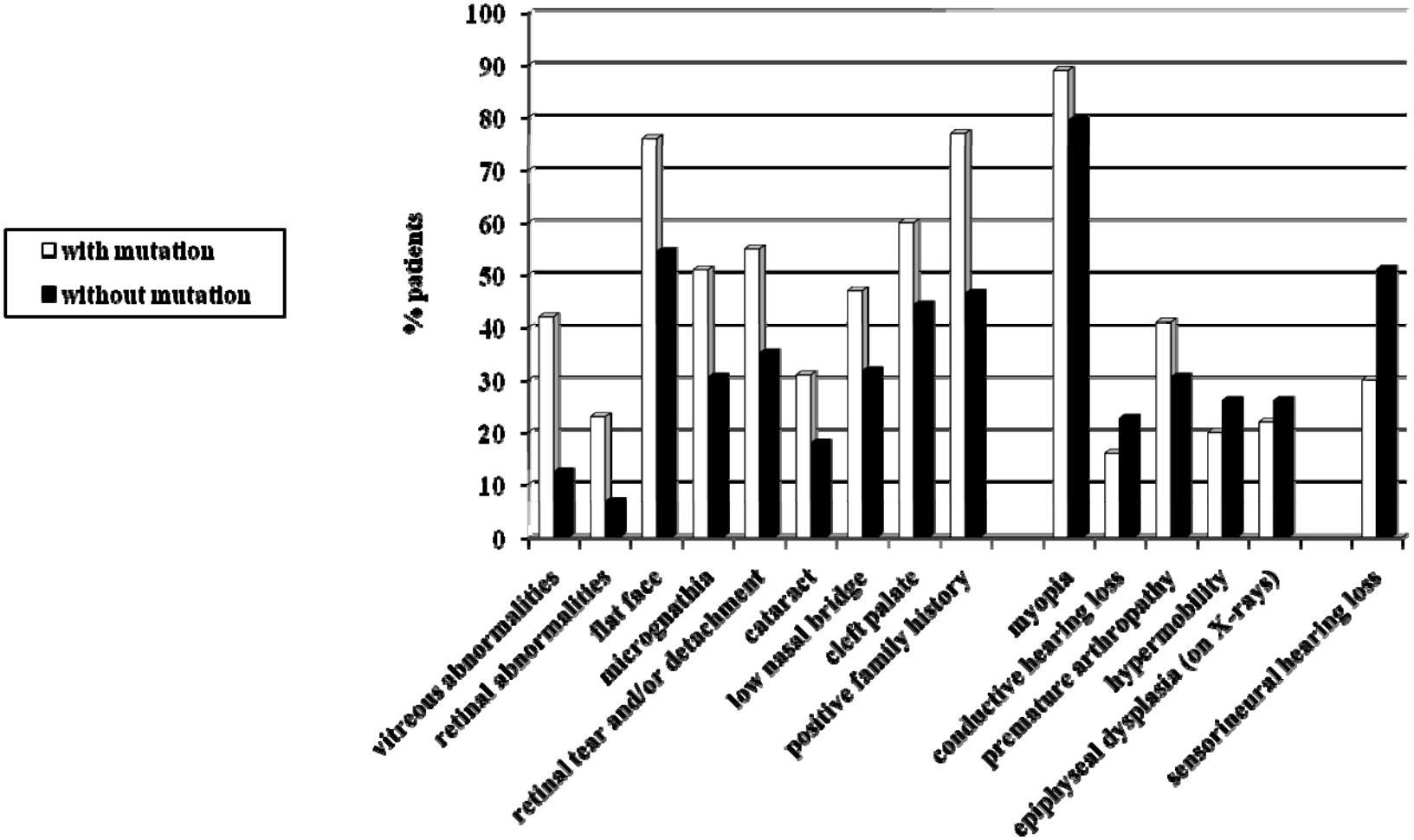

Figure 1 –Frequency of clinical and radiographic characteristics in patients with a COL2A1 mutation (white bars) and patients without a COL2A1 mutation (black bars). From left to right: the first 9 characteristics have a p-value ≤ 0.05, the following 5 characteristics are not statistically significant, the remaining characteristic (sensorineural hearing loss) shows reverse significance with p-value < 0.005

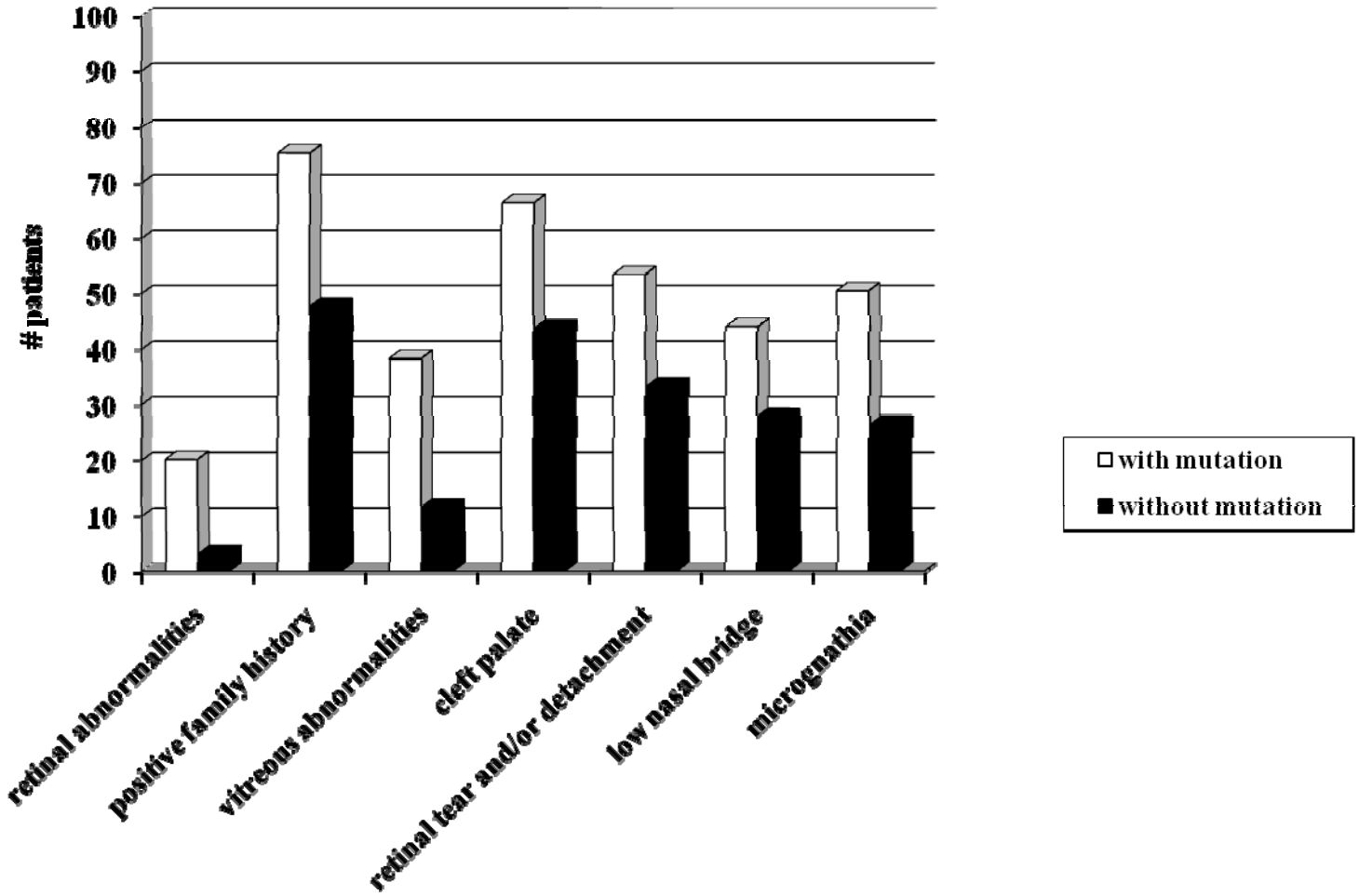

Figure 2 – Frequency of the 7 most distinguishing characteristics in both the mutation negative and mutation positive group

Table 2: Proposed scoring system

Characteristics	Score
- retinal abnormalities	5
- positive family history	5
- vitreous abnormalities	4
- cleft palate	4
- retinal tear and/or detachment	3
- low nasal bridge	1
- micrognathia	1
Total score	23

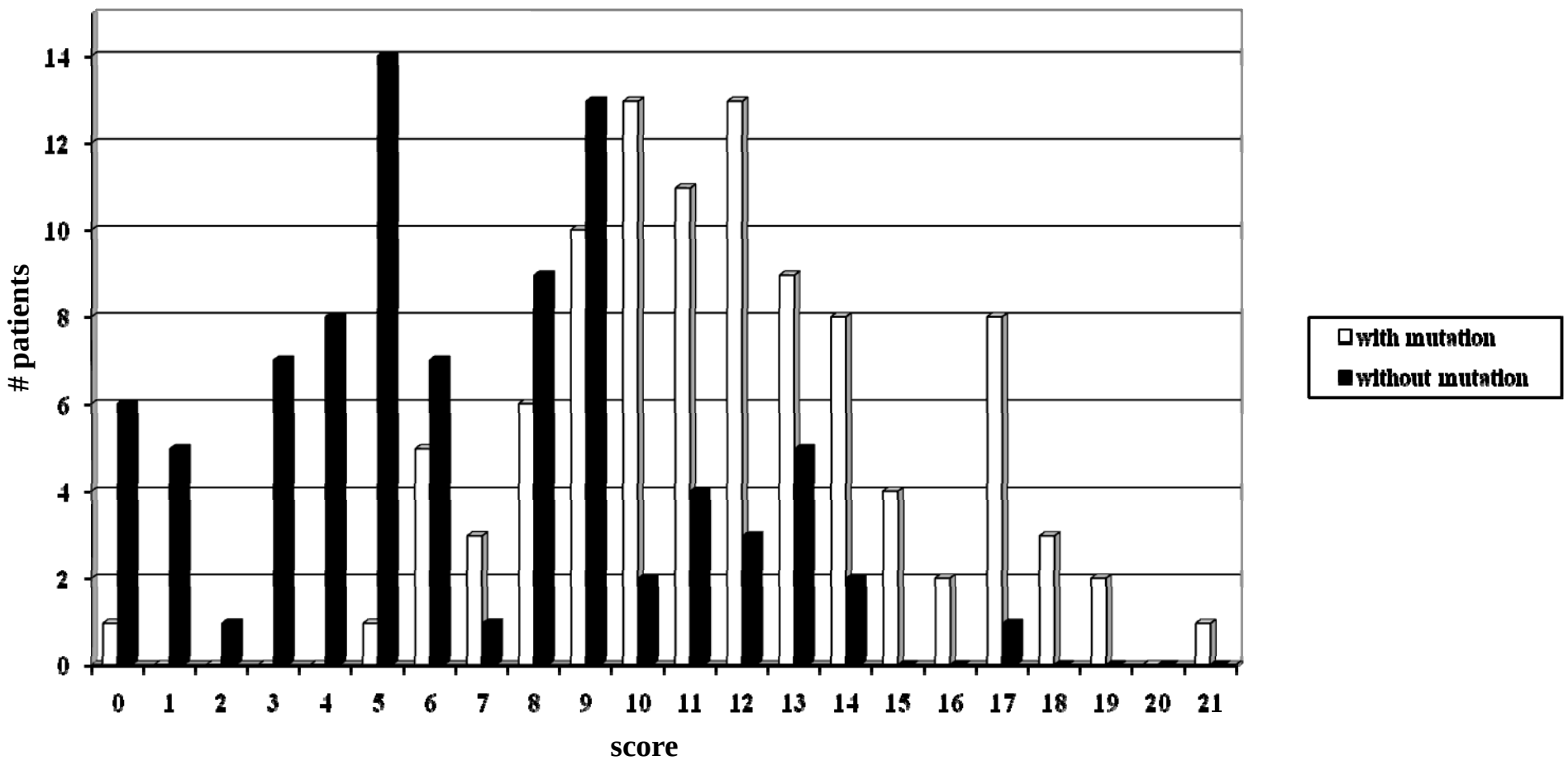

Figure 3 – Overlap in total score between mutation positive and mutation negative group of patients.

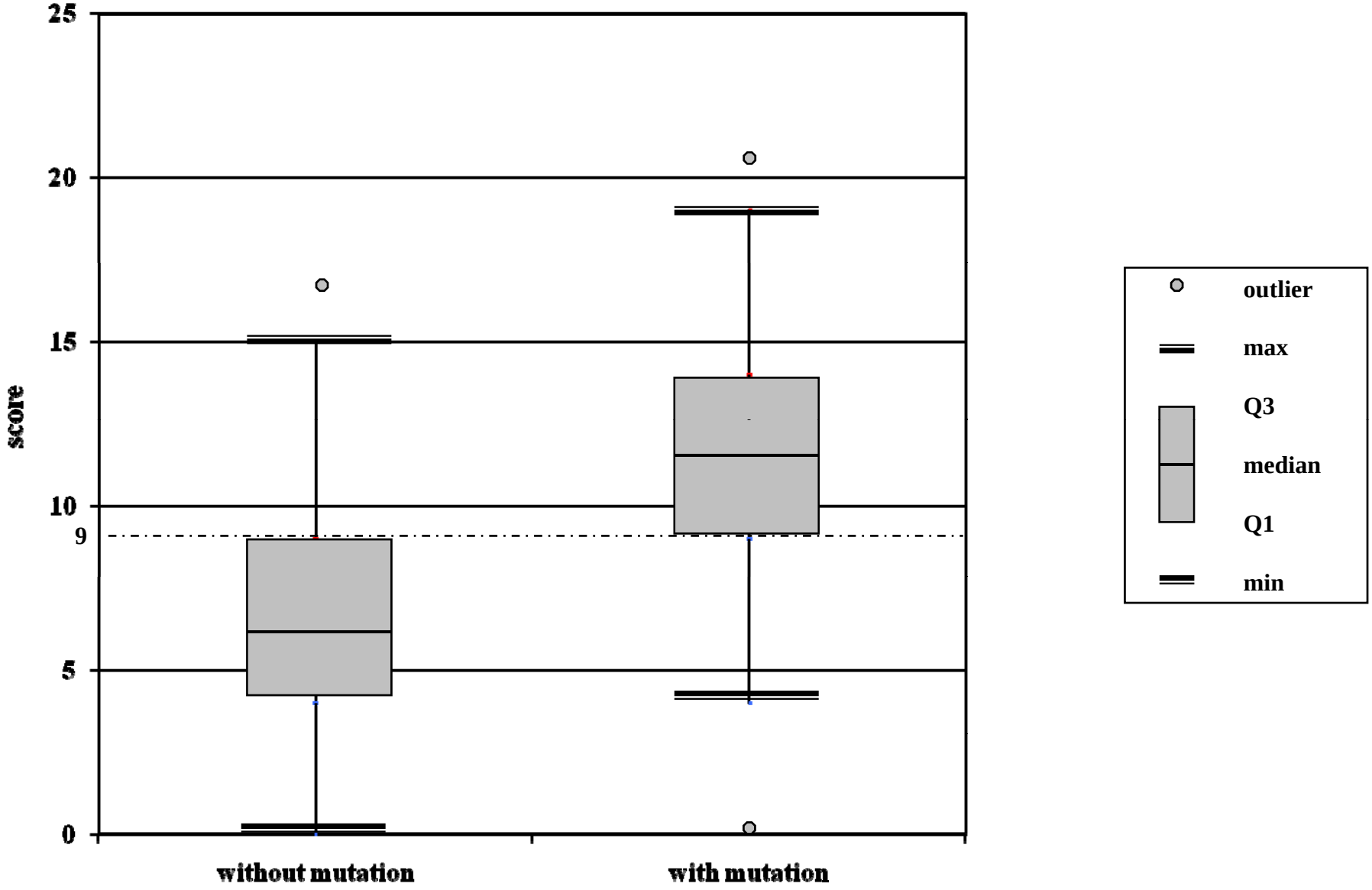

Figure 4 – Box plot presentation of the total scores in both patient groups with Q1 representing the first quartile or 25th centile and Q3 representing the third quartile or 75th centile. Max indicates the maximum score, and min the minimum score, that is not an outlier or that is within 1.5 times the interquartile range (Q1-Q3). 75% of the patients with a *COL2A1* mutation had a total score ≥ 9