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Reclassification of variants of uncertain significance in type 1 collagen genes: a national reference laboratory experience

Short Title: Variants of uncertain significance in Osteogenesis Imperfecta

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ABSTRACT

The availability of large volumes of data from genetic testing has enabled the interpretation of more DNA variants, contributing to a greater number of identified variants of uncertain significance (VUS). The growing number of VUS causes a burden of inconclusive findings in clinical practice. Osteogenesis imperfecta (OI) is a genetically heterogeneous connective tissue disorder causing bone fragility and limb deformity. Pathogenic variants in two collagen genes, *COL1A1* and *COL1A2* account for around 90% of all OI. Data mining of the variants from Sheffield Diagnostic Genetics Service (SDGS), the national OI testing hub (UK) was conducted to collate all VUS in *COL1A1* and *COL1A2* identified. All VUS were then reclassified according to the latest 2024 ACGS best practice guidelines. A total of 161 VUS in *COL1A1* and 98 VUS in *COL1A2* were identified and reanalysed. For *COL1A1*, we found that 2% VUS were upgraded to likely pathogenic, 23% of the VUS were downgraded to likely benign and benign, 12% were reclassified as hot VUS and the remaining 63% have not changed classification as VUS. With regards to *COL1A2*, only 1% of the VUS were upgraded to likely pathogenic, 25% were downgraded to likely benign and benign, 13% were reclassified as hot VUS and 61% remained as VUS. From this study, we demonstrated that iterative reanalysis of VUS is crucial in clinical practice as new data and evidence become available. This dynamic process will significantly improve diagnostic accuracy and inform patient care decisions.

Keywords: type 1 collagen genes, Variants of uncertain significance (VUS), reclassification

- What is already known on this topic

  Reclassification of variants is critical to ensure prompt diagnosis of rare disease with an ever-changing landscape of genomic testing.
- What this study adds

Currently, more than 60% of variants remain as VUS, emphasising the urgent need for additional functional and clinical data, transforming uncertain genetic findings into clinically and scientifically actionable insights.

- How this study might affect research, practice or policy

  Iterative, dynamic assessment of VUS especially for clinically treatable conditions such as Osteogenesis Imperfecta are required to harness the advances of genomic medicine in clinical practice.

INTRODUCTION

Fibrillar collagens are the most abundant protein structure in vertebrates. They consist of major types I, II, III as well as minor types V and XI. Type I collagen serves as the primary protein in the extracellular matrix of bone and skin (1). Pathogenic variants in the genes responsible for type I procollagen are linked to various disorders, including autosomal dominant osteogenesis imperfecta (OI). OI is a genetically heterogenous disorder that is caused by variants in approximately 20 different genes. OI represents a range of conditions, from mild to life-threatening, characterised by differing degree of bone fragility, increased risk of fractures, short stature, blue sclerae, hearing impairments, and dental anomalies like dentinogenesis imperfecta (2). Defect in type 1 collagen, encoded by *COL1A1* and *COL1A2* account for around 90% of OI patients (3). On the contrary, recessive forms of OI are linked to pathogenic variants in genes involved in collagen processing and post-translational modification. For example, OI Type 10 arises from mutation in *SERPINH1* which disrupt collagen folding and cross-linking (4). Alternatively, OI Type 19 is inherited by X-linked recessive form that is caused by mutation in the *MBTPS2* (5).

The rapid advancements in genomic testing have significantly improved diagnostic accuracy and efficiency with more genomic variants being identified. However, this also brings the challenge of managing an increasing number of VUS, the genomic variants that are not

classified as either pathogenic or benign due to insufficient evidence to confirm their pathogenicity (6). In the absence of supporting clinical data and limited functional evidence available, many variants remain classified as uncertain significance which can complicate interpretation in clinical settings. This contributes to increasing uncertainty in determining variant pathogenicity.

When new evidence is available, the need for reinterpretation of VUS is necessary (7). Nevertheless, most diagnostic laboratories do not perform reclassification on a regular basis due to the significant time and labour involved. Variant reclassification is carried out upon clinicians' requests when there is a new referral or change in clinical presentation. Reclassification of VUS remain a dynamic process, influenced by various factors, including access to new functional studies and clinical data.

VUS can be further classified into subgroups reflecting their likelihood of causing disease, with probabilities ranging from 10% to 90%. This subclassification uses a temperature system: VUS with a 10-32.5% probability are "ice cold" and "cold"; those with a 32.5-67.5% probability are "cool" and "tepid"; and VUS with a 67.5-90% probability are labelled "warm" and "hot" (8). Critically, Association for Clinical Genomic Science (ACGS) best practice guidelines recommended only hot VUS can be reported to the clinicians. This VUS subgroup has potential to be upgraded to likely pathogenic when additional evidence is available. Although subclassifications of VUS can be helpful for internal discussions amongst clinical scientists and clinical geneticists, these terms should not be included in the formal patient report to avoid confusion or unnecessary concern (9).

In this current study, we aimed to collate a dataset of VUS in two collagen genes, *COL1A1* and *COL1A2* from the database at Sheffield Diagnostic Genetics Service (SDGS), the national OI testing hub and reclassify the VUS using current, updated variant classification guidelines.

The purpose of this project is to examine the value of reclassification of VUS for improving patient diagnostic rate.

MATERIALS AND METHODS

Study population

This is a retrospective cohort study of National Health Service (NHS) patients with documented genetic testing for OI conducted at SDGS that serves as the national OI testing centre in England.

DNA extraction and sequencing

Peripheral blood samples were collected from the patients and their parents. The QIAmp DNA Blood Midi kit (Qiagen, Venlo, The Netherlands) was used to extract the DNA from these samples.

The samples where then prepared for analysis using Next Generation Sequencing (NGS). Osteogenesis Imperfecta autosomal dominant and autosomal recessive panels, R102 Gene Panel (https://panelapp.genomicsengland.co.uk/panels/196/) were used consisting of: NM_006129.5 (*BMP1*); NM_000088.3 (*COL1A1*); NM_000089.3 (*COL1A2*); NM_052854.3 (*CREB3L1*); NM_006371.4 (*CRTAP*); NM_001025295.1 (*IFITM5*); NM_022356.3 (*P3H1*); NM_000942.4 (*PPIB*); NM_021939.3 (*FKBP10*); NM_152860.1 (*SP7*); NM_002615.4 (*SERPINF1*); NM_001235.2 (*SERPINH1*), NM_182943.2 (*PLOD2*); NM_ 018112.1 (*TMEM38B*); NM_005430.3 (*WNT1*), NM_000918.2 (*P4HB*); NM_005032.6 (*PLS3*); NM_014822.2 (*SEC24D*); NM_003118.3 (*SPARC*); NM_153365.2 (*TAPT1*); NM_022167.3 (*XYLT2*).

Library preparation

The Covaris E220 sonicator was used for the shearing of genomic DNA. The SureSelectCT library system (Agilent Technologies) was used to perform the end repair, A tailing and ligation of adaptors. SureSelect target enrichment (Agilent Technologies) performed target enrichment using custom in house designed probes. Sequencing was performed on the Illumina HiSeq using the HiSeq Rapid SBS Kit v2 performing 2 × 108 base pair paired end reads.

Data analysis

Data analysis was carried out using the Best Practice Guidelines from Broad Institute. Burrows-Wheeler Aligner (BWA) alignment was used to map the reads to the human reference sequence (GRCH37/hg19) (http://www.broadinstitute.org/gatk/guide/best-practices). A read depth of 30-fold was set as a minimum threshold for exonic sequences and intronic sequences up to and including 5 bp from the ends of each exon. A read depth of 18-fold was set as the minimum threshold got intronic sequences from 6 to 25 bp from the ends of each exon. Haplotype Caller (Broad Institute) was used to identify variants, and the variants were filtered against this in house polymorphism list.

Variant reporting

Once identified, the variants were compared to cDNA reference sequences NM_003118.3 and then assessed with Alamut Visual version 2.11 QT v5.5.1 (Interactive Biosoftware, Rouen, France). The sequence variants were all classified using ACMG/AMP guidelines for variant interpretation (10) and ACGS Best Practice Guidelines for variant classification that was implemented in the UK in 2018 (11). The ACGS guidelines refine the ACMG/AMP framework with UK-specific adaptations by introducing quantitative scoring of evidence for each category:

(very strong = 8, strong = 4, moderate = 2, supporting = 1) in addition to the ACMG criteria for classifying variants as simplified in Table 1.

Table 1. Description of ACMG criteria

Category	Description
Pathogenic criteria	
PVS1	Null variant in gene with known loss-of-function mechanism
PS1	Same amino acid change as known pathogenic variant
PS2	Confirmed *de novo* variant in affected individual
PS3	Well-established functional studies show damaging effect
PS4	Variant prevalence in affected individuals is significant
PM1	Variant located in mutational hotspot or functional domain
PM2	Absent or rare in population databases
PM3	Variant detected in trans with a pathogenic variant (recessive)
PM4	Protein length changes due to in-frame indels
PM5	Novel missense change at the same residue as pathogenic
PM6	Assumed de *novo* without confirmation
PP1	Co-segregation with disease in multiple affected family
PP2	Missense variant in gene with low benign missense rate
PP3	Multiple computational tools predict deleterious effect
PP4	Patient phenotype highly specific for disease
PP5	Reputable source reports pathogenicity
Benign criteria	
BA1	Allele frequency too high for disorder
BS1	Allele frequency greater than expected for disorder
BS2	Observed in healthy individuals
BS3	Functional studies show no damaging effect

BS4	Lack of segregation in affected family members
BP1	Missense variant in gene where loss-of-function causes disease
BP2	Variant observed in trans with pathogenic variant (dominant)
BP3	In-frame indel in non-conserved region
BP4	Computational tools predict benign effect
BP5	Variant found in case with alternate cause
BP6	Reputable source reports benign status
BP7	Synonymous variant with no splicing impact

VUS reanalysis

A database of previously reported VUS in *COL1A1* and *COL1A2* were collated from the laboratory records. All VUS records until February 2024 were retrieved and included in this study. Variant reanalysis was conducted by using ClassIC, a consolidated database used by North East & Yorkshire Genomic Laboratory Hub (NEY-GLH). There are several databases such as ClinVar, Human Gene Mutation Database (HGMD) and Genome Aggregation Database (gnomAD) and *in-silico* prediction tools; Rare Exome Variant Ensemble Learner (REVEL) and a combination of splice site predictive programs that were utilised throughout the process to collect evidence for reinterpretation. Reclassifications were defined as an upgrade when the variant was moved to a more severe category and a downgrade when the variant was reclassified to a less severe category (12).

RESULTS

Cohort summary

From the SDGS record, 753 variants were identified in *COL1A1* and 418 in the *COL1A2* as shown in Table 2 with more than 50% were likely pathogenic (LP) and pathogenic (P) and approximately 20% were classified as VUS. A remaining small percentage of the total variants were reported as likely benign (LB) and benign (B).

Table 2. Variants in *COL1A1* and *COL1A2*

Classification/Gene	*COL1A1*	*COL1A2*
Pathogenic	301	144
Likely pathogenic	162	84
VUS	161	98
Likely benign	80	53
Benign	49	39
Total variant	**753**	**418**

Full analysis of 32 different genes associated with OI was performed using R102 Gene Panel. In another study, for 4 years period from 2020 to 2024, there were 935 referral diagnostic cases of OI and about 42% of total cases have reported pathogenic variants. Approximately 90% of the pathogenic variants were found in *COL1A1* and *COL1A2*, while in 17 of 32 genes had no reported pathogenic variants as presented in Figure 1 here (unpublished data, SDGS 2024).

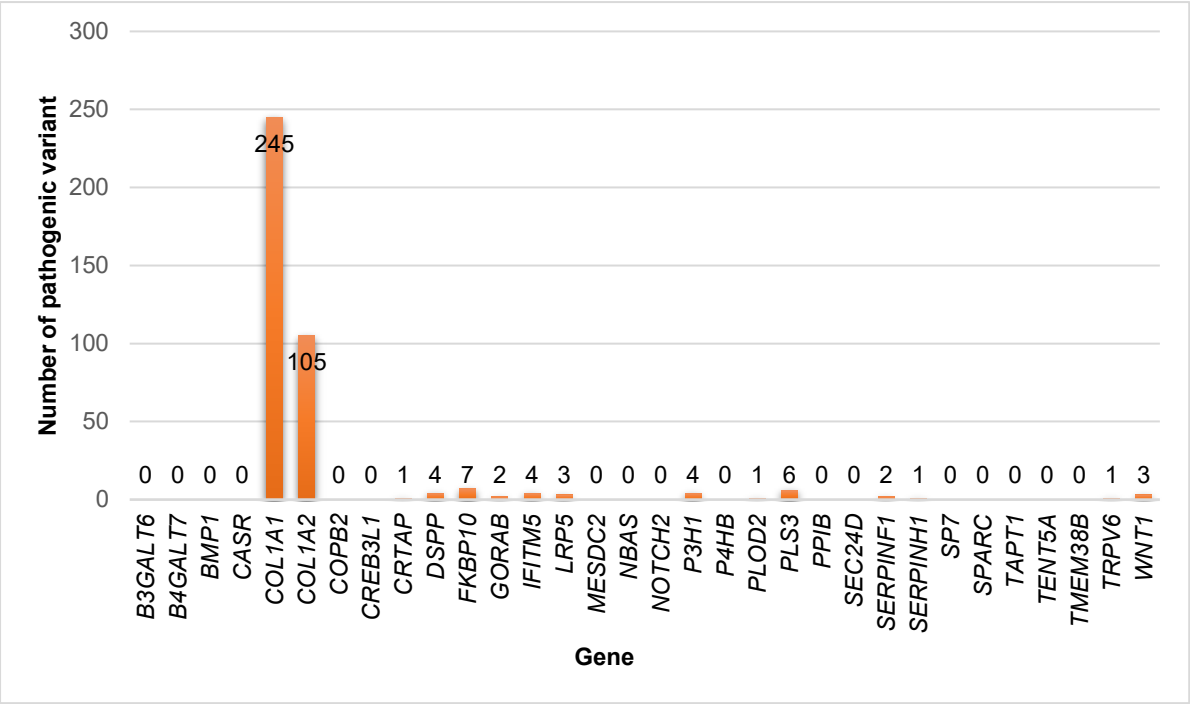

Figure 1. Number of pathogenic variant in different genes tested for OI for year 2020 to 2024. Of the 386 pathogenic variants identified, 350 were in type 1 collagen genes.

Variant reclassification

To re-evaluate the classification of VUS, ACMG and ACGS guidelines were applied for each identified variant. A total of 161 VUS in *COL1A1* and 98 VUS in *COL1A2* were identified and reanalysed. These VUS are widely distributed along the gene including the collagen triple helix domain, fibrillar collagen C-terminal propeptide domain and intronic region.

Different types of DNA variants in our VUS data are summarised in Table 2 with 42% in *COL1A1* and 55% in *COL1A2* are missense variants, about 27% are splice region variants and the smaller percentage of in-frame deletions, duplications, insertions, synonymous and intronic variants. Splice region variants that were described here are the sequence changes that occur near exon boundaries, specifically in the donor motif (last 3 bases of the exon and adjacent 3–6 intronic nucleotides) or the acceptor motif (first base of the exon and 3-20

nucleotides upstream of the exon boundary) (13). There are also 5 non-coding variants located at 5' untranslated region (5' UTR) and 3' UTR region found in *COL1A1*.

Table 2. Types of variant for VUS in *COL1A1* and *COL1A2*.

Types of DNA variant	*COL1A1*	*COL1A2*
Missense	67	54
Deletion	7	2
Duplication	6	3
Insertion	2	0
Synonymous	13	5
Splice region	45	26
Intronic	16	8
Non-coding	5	0
Total	161	98

Of 161 VUS in *COL1A1*, 41 were reclassified (25%); 4 were upgraded to likely pathogenic (2%), and 37 were downgraded to benign or likely benign (23%). In contrast, from 98 VUS in *COL1A2*, only 1 was upgraded to likely pathogenic (1%), and 24 were downgraded to benign or likely benign (24%). Our findings also show that 101 out of 161 VUS in *COL1A1* and 59 from 98 VUS in *COL1A2* have not changed classification, accounting for more than 60% of the total variants that have been reanalysed. This highlights the lack of evidence to upgrade or downgrade the initial classification. The remaining 18 VUS in *COL1A1* and 10 VUS in *COL1A2* have now been reclassified as hot VUS, assigned a score of 5 points of evidence level and detailed in Supplementary Table 1.

VUS upgrade to likely pathogenic

Table 3 summarizes the criteria employed to reclassify VUS, highlighting the evidence used to support their upgrade to likely pathogenic.

Table 3. List of VUS which were upgraded to likely pathogenic

Gene	Variant	Criteria used	New classification
COL1A1	c.4327G>A	PM2, PM1 (supporting), PP3, PM5 (supporting), PS4 (supporting)	LP
COL1A1	c.4328C>T	PM2, PS4 (moderate), PP3, PM1 (supporting)	LP
COL1A1	c.3150_3158dup	PS4 (moderate), PM4, PM2	LP
COL1A2	c.2031_2048dup	PM2, PM4, PS4 (moderate)	LP

We present here a reported VUS in *COL1A1* that has been upgraded to likely pathogenic (LP). A missense variant c.4328C>T was reported in one patient in our database. This variant was initially assigned as a VUS with no specific criteria assigned as this was classified before 2015. During the reclassification, this variant was absent from the frequency database, gnomAD v4.1.0 therefore we applied PM2 moderate criteria. PM1 supporting was assigned as it is located at the C-terminal propeptide domain with few other missense variants reported to be likely pathogenic and pathogenic within the region. The same VUS was reported in individual(s) with moderate to severe clinical features of OI by previous literature (14). They reported this variant as likely pathogenic (PM2, PM6, PP3, PP4 criteria). PM6 criteria was applied in this study; however, due to the limited information available, the same criteria was not utilised for our reclassification. Next generation sequencing (NGS) on a large population of Chinese patients with OI reported this missense change has been observed in individual(s) with clinical features of OI Type III (15). Another patient with OI Type IV was reported to have

the same variant in a more recent study on a large cohort of OI patients (16). This is suggestive of the application of PS4 moderate. PP3 supporting was also assigned based on the REVEL score of 0.826.

Another VUS located at neighbouring position to the previously mentioned variant at location c.4327G>A was reclassified as likely pathogenic. This missense variant was detected in three individuals, two of whom are members of the same family in our database. In previous classification, PP3 supporting, based on REVEL score of 0.871 and PM2 moderate as the variant was not present in normal population from our gnomAD v.4.1.0 search criteria were assigned. PM1 supporting was also assigned to this VUS as it was located at C-terminal propeptide domain that is essential for the correct folding of –chains prior to the triple helix formation (17). Additionally, in one individual, the variant was inherited from an unaffected father who carries the variant. Two new criteria supported its pathogenicity of it including PM5 moderate, as it represents a novel missense change at the same amino acid residue with the mentioned variant above. We also applied PS4 supporting criteria to this variant as it was also identified in a second unrelated OI patient. The classification of these two variants has been upgraded, and revised reports have been provided to clinicians.

Factor contributing to downgrade from VUS to Likely Benign / Benign

This study also demonstrates that approximately 20% of VUS were now downgraded to either likely benign or benign. Details of each variant that are now downgraded to likely benign or benign is available in Supplementary Table 2. The availability of gnomAD database which is more extensive allow us to identify many variants that are now commonly detected in general normal population. The first gnomAD dataset was published in 2019, in which earlier analyses had relied on resources such as the Exome Aggregation Consortium (ExAC) and NCBI frequency data. This transition provides a more comprehensive and diverse reference for

variant interpretation, incorporating not only allele frequency data but also variant co-occurrence and population-level patterns (18).

DISCUSSION

In this current study, we focused to reclassify variants in *COL1A1* and *COL1A2* aiming to improve the patient diagnostic throughput. Resource and time constraints makes us focus on targeting genes that are most likely to be impactful. Although numerous new genes linked to OI have been identified, each of these accounts for fewer than 10% of the cases reported to date in comparison to type 1 collagen genes. It is less likely that there would be new information available for reclassification. We did not have national guidelines before introduction of ACMG recommended guidelines ones were beginning to be used from 2018. Therefore, as tools and guidelines changed the classification of variants expected to change.

Our analysis identified 26 VUS in the *COL1A1* and 13 VUS in *COL1A2* located within the collagen triple helix repeat domain. Additionally, a comparable number of VUS were observed in the C-terminal propeptide domain, with 28 in *COL1A1* and 16 in *COL1A2* including the upgraded variants explained above. The clustering of these variants suggests the potential functional significance to pathogenicity.

Glycine substitutions, particularly those occurring within the collagen triple helix domain are widely recognised to play critical role in maintaining the structural integrity of type I collagen. They are often classified as likely pathogenic or pathogenic. For the non-glycine substitutions, we assumed that the position of variants along the gene could influence their potential to cause disease. As demonstrated in two variants from this study, we anticipated that VUS found in this C-terminal propeptide protein domain could potentially be upgraded, provided the availability of additional evidence. We recommend conducting functional studies on these

VUS, particularly the hot VUS, which required just one additional piece of evidence for reclassification as likely pathogenic.

There are few pathogenic variants have been reported at the neighbouring region of the upgraded VUS in *COL1A1* reported here. Notably, a glycine substitution at c.4343G>A (p.Gly1448Asp) was reported as likely pathogenic for OI, supporting the potential significance of nearby variants (17). Another pathogenic non-glycine substitution at c.4321G>C (p.Asp1441His) has been reported in individuals with osteogenesis imperfecta. In at least one individual, this variant was identified as *de novo* (19). The pathogenic variants detected near the upgraded VUS may provide additional evidence for the reclassification of VUS in these regions.

In contrast, the upgraded VUS in *COL1A2* located within the collagen triple helix repeat domain. ClinVar records indicate multiple pathogenic variants have been reported in this domain including frameshift variants and glycine substitutions, which were classified as either likely pathogenic or pathogenic (20). The recurrence of the pathogenic variants in the same region reinforces the clinical relevance of this domain and supports the reclassification of nearby VUS, particularly when they affect conserved residues or structurally critical domains.

When reviewing the literature on type 1 collagen genes, there was lack of studies focusing on VUS reclassification. Generally, reclassification occur years after the initial genetic testing (21). This emphasises the importance of ongoing monitoring by both clinicians and patients for any updates to the classification. A recent publication proposed a structured framework approach for routine reinterpretation of variants, emphasising the need to establish appropriate infrastructure, secure funding for reclassification efforts, implement systems to manage updates for clinicians and notify patients, and ensure patient consent is obtained (22).

Limitation of this study includes the extended duration required, that may span several months to complete reclassification process. We introduced a collaborative approach between Sheffield Children's NHS Foundation Trust and The University of Sheffield, facilitated through the Julia Garnham Centre, to address NHS case backlogs. This centre provides students and scientists with valuable opportunities to learn and gain experience in genomic analysis. The establishment of the Julia Garnham Centre has proven effective in alleviating case backlogs. Students conduct low-resolution analyses, which are subsequently verified by NHS genomic scientists.

Another challenge identified was the complexity of the existing guidelines. While guidelines for sequence variants are accessible, their interpretation often varies across different laboratories. We observed challenges with the current guidelines and propose the need for specific recommendations tailored to the interpretation of variants for collagen related disorders.

Reclassification of sequence variants is a dynamic process therefore needs to be done regularly. With the updated and specific classification guidelines and availability of new evidence, we may find these variants to be upgraded or downgraded from time to time. This is very critical for clinical evaluation and patient care.

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AUTHOR CONTRIBUTIONS

Nurhaziqah Supari: Formal analysis, Writing - original draft. Duncan Baker: Supervision, Writing - original draft, review & editing. Sylvia Keigwin: Formal analysis, Writing – review &

editing. Sophie Delaney: Formal analysis, Writing – review & editing . Seiko Makino: Formal analysis. Meena Balasubramanian: Supervision, Writing - review & editing.

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ETHICAL APPROVAL

This study was approved by Research & Innovation at Sheffield Children's NHS Foundation Trust.

COMPETING INTERESTS

The authors declare no competing interests.

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