Neuropathology and Applied Neurobiology

WILEY

Neuropathology and Applied Neurobiology
JOURNAL OF THE BRITISH NEUROPATHOLOGICAL SOCIETY

ORIGINAL ARTICLE OPEN ACCESS

Collablots: Quantification of Collagen VI Levels and Its Structural Disorganisation in Cell Cultures From Patients With Collagen VI-Related Dystrophies

Nadia Osegui-Barcenilla[1] | Maria Sendino[1] | Sergio Martín-González[1] | Itziar González-Moro[1] | Ainhoa Benito-Agustino[1] | Noemi Torres-Conde[1] | Andrea López-Martínez[1] | Cecilia Jiménez-Mallebrera[2,3] | Arístides López-Márquez[2,3,4] | Virginia Arechavala-Gomeza[1,5]

[1]Nucleic Acid Therapeutics for Rare Disorders (NAT-RD), Biobizkaia Health Research Institute, Barakaldo, Spain | [2]Laboratory of Applied Research in Neuromuscular Diseases, Neuromuscular Pathology Unit, Neuropediatric Service, Institut de Recerca Sant Joan de Déu, Esplugues de Llobregat, Spain | [3]Rare Diseases Network Biomedical Research Center (CIBERER), Madrid, Spain | [4]Department of Genetics, Microbiology and Statistics, Faculty of Biology, University of Barcelona, Barcelona, Spain | [5]Ikerbasque, Basque Foundation for Science, Bilbao, Spain

Correspondence: Virginia Arechavala-Gomeza (v.arechavala@live.co.uk)

Received: 6 December 2024 | **Revised:** 29 April 2025 | **Accepted:** 13 May 2025

Funding: This work was supported by the Fundación Noelia and from grant PID2021-125041OB-I00 funded by the MICIU/AEI/10.13039/501100011033 and by the ERDF/EU. A.L-M acknowledges funding from the FPU Programme of the Spanish Ministry of Science, Research and Universities (FPU21/00912). V. A.-G. acknowledges funding from Ikerbasque, (Basque Foundation for Science). C. J.-M. and A. L.-M. acknowledge funding as well from Fundación Noelia and the Instituto de Salud Carlos III (PI22/01382). A. L-M holds a research grant from Alexion, AstraZeneca Rare Disease.

Keywords: Bethlem myopathy | collagen type VI | congenital muscular dystrophy | diagnostic techniques and procedures | immunoblotting, western | immunocytochemistry | quantitative evaluation | Ullrich congenital muscular dystrophy

ABSTRACT

Aims: This study aims to develop a quantitative method for assessing collagen VI expression in cell cultures, which is crucial for the diagnosis and treatment of collagen VI-related dystrophies.

Methods: We developed a combined in-cell western (ICW) and on-cell western (OCW) assay, which we have called 'collablot', to quantify collagen VI and its organisation in the extracellular matrix of cell cultures from patients and healthy controls. To optimise it, we optimised cell density and the protocols to induce collagen expression in cultures, as well as the cell fixation and permeabilisation methods. This was completed with a thorough selection of collagen antibodies and a collagen-hybridising peptide (CHP). We then used collablots to compare cultures from patients and controls and evaluate therapeutic interventions in the cultures.

Results: Collablots enabled the quantification of collagen VI expression in both control and patient cells, aligning with immunocytochemistry findings and detecting variations in collagen VI expression following treatment of the cultures. Additionally, CHP analysis revealed a marked increase in collagen network disruption in patients compared to the controls.

Conclusions: The collablot assay represents a suitable method for quantifying collagen VI expression and its organisation in culture and assessing the effect of therapies.

Abbreviations: COL6-RD, collagen VI-related dystrophies; BM, Bethlem myopathy; UCMD, Ulrich congenital muscular dystrophy; ECM, extracellular matrix; IHC, immunohistochemistry; ICC, immunocytochemistry; WB, western blot; OCW, on-cell western; ICW, in-cell western; FC, flow cytometry; CHP, collagen-hybridising peptide.

Summary

- Evaluating therapies for collagen VI-related dystrophies (COL6-RD) requires the quantification of collagen VI levels.
- Collablot assays are a novel method for quantifying collagen VI expression and its structural organisation in cell culture.
- Due to the significant role of phenotypic heterogeneity in this complex disease, quantifying collagen alone might not be adequate for diagnosing COL6-RD, but the addition of a peptide to quantify collagen disorganisation could help characterise patient cultures.

1 | Introduction

Myopathies associated with collagen VI deficiency are caused by one or more pathogenic variants in the *COL6A1*, *COL6A2* and/or *COL6A3* genes, which encode the three essential α-chains of collagen VI. These variants result in a spectrum of diseases known as collagen VI-related dystrophies (COL6-RDs), ranging from relatively mild Bethlem muscular dystrophy (BM) to severe Ullrich congenital muscular dystrophy (UCMD). Intermediate phenotypes, referred to as intermediate COL6-RD, represent the clinical spectrum observed between the two extremes previously mentioned [1].

In muscle, collagen VI functions as a ubiquitous extracellular matrix (ECM) protein within the stroma, constructing a microfibrillar network linked to the basement membrane [2]. It is synthesised not by myocytes but by the resident primary interstitial muscle fibroblasts in the musculoskeletal system [3]. Inside these fibroblasts, the three distinct chains come together to form trimeric structures, subsequently organised into antiparallel dimers and eventually assembled into tetramers. These tetramers are secreted into the extracellular matrix to form the previously mentioned microfibril network [4] (Figure 1A).

Variants in the genes encoding essential chains disrupt trimer formation, causing intracellular collagen retention and impaired microfibril network formation, albeit through different mechanisms. Loss of function (e.g., premature termination) always needs a second variant to be disease-causing [1]. These variants may reduce the amount of collagen VI in the ECM while ensuring the correct organisation of the network (Figure 1B). Conversely, dominantly acting variants (e.g., missense variants) are more likely to induce structural changes that impair trimer assembly (Figure 1C) [5].

While formal diagnostic criteria for collagen VI-related dystrophies have not yet been established, diagnosis typically involves clinical evaluation, muscle biopsy analysis and identification of variants in the relevant genes using molecular genetic testing [1]. However, achieving a genetic diagnosis can be challenging due to the large size of the *COL6A* genes (*COL6A1*: 23,281 bp, *COL6A2*: 34,737 bp, *COL6A3*: 40,493 bp) and their high frequency of polymorphisms. Despite being invasive, muscle biopsies remain the sole method to assess collagen expression, albeit qualitatively.

Several approaches have been proposed to genetically treat collagen VI-related dystrophies, with most strategies focusing on RNA molecules or gene editing techniques [6–9]. However, to assess the efficacy of these putative therapies, there is currently no validated method for quantifying collagen expression in the cell cultures that are routinely used to develop these treatments.

Efforts have been made to develop methods for studying collagen VI expression, but most lack quantitative accuracy. The primary method for assessing this protein in patients' muscles is direct labelling of muscle biopsies with antibodies against collagen VI; this approach can localise and show a decrease in the expression of collagen VI. However, obtaining muscle biopsies is often challenging due to their invasive nature. In such cases, immunolabelling studies are sometimes performed on skin biopsies. Both methods exhibit high sensitivity and specificity in diagnosing Ullrich muscular dystrophy (close to 100%), with lower specificity for Bethlem myopathy (63%). Therefore, while collagen VI immunostaining is a valuable diagnostic tool, particularly for Ullrich cases, it is not a quantitative technique and heavily relies on the expertise of the pathologist analysing the sample [10, 11].

In vitro studies can also be conducted using skin-derived fibroblasts. Conventional analysis of collagen VI typically involves immunocytochemistry, a method that can reveal a reduction or abnormal deposition of the extracellular matrix of collagen VI, along with increased intracellular retention observed upon cell permeabilisation. However, this method is both laborious and non-quantitative [12]. Another *in vitro* analysis using fibroblasts is flow cytometry, which provides quantitative data but requires a large number of cells, which are often difficult to obtain or require prolonged culture periods [13].

We have prior experience in developing methods to quantify proteins associated with other neuromuscular disorders using in-cell western (ICW) technology [14, 15]. An in-cell western is a quantitative immunofluorescence assay performed in microplates, allowing for the quantification of proteins directly within cell cultures. Collagen VI is a protein produced intracellularly but is excreted and organised in the extracellular matrix. To accurately quantify this protein, we have combined ICWs, which target intracellular proteins thanks to cell permeabilisation, with on-cell westerns (OCW), where samples are not permeabilised, hence staining only the secreted portion of the protein. Additionally, we assessed the integration of a ligand to measure collagen disorganisation within the same system: collagen-hybridising peptides (CHPs) demonstrate high binding specificity to denatured collagen chains while exhibiting very low affinity for intact (triple-helical) collagen [16]. We have optimised this assay, which we call 'collablot', to effectively evaluate variations in protein expression in patient cultures and provide an *in vitro* screening method for potential therapies.

2 | Material and Methods

2.1 | Samples

Primary fibroblasts were used to refine and evaluate the protocol. These cells, derived from skin biopsies of healthy controls and COL6-RD patients collected after informed consent (Table 1), were either purchased from commercial suppliers (CTRL-2,

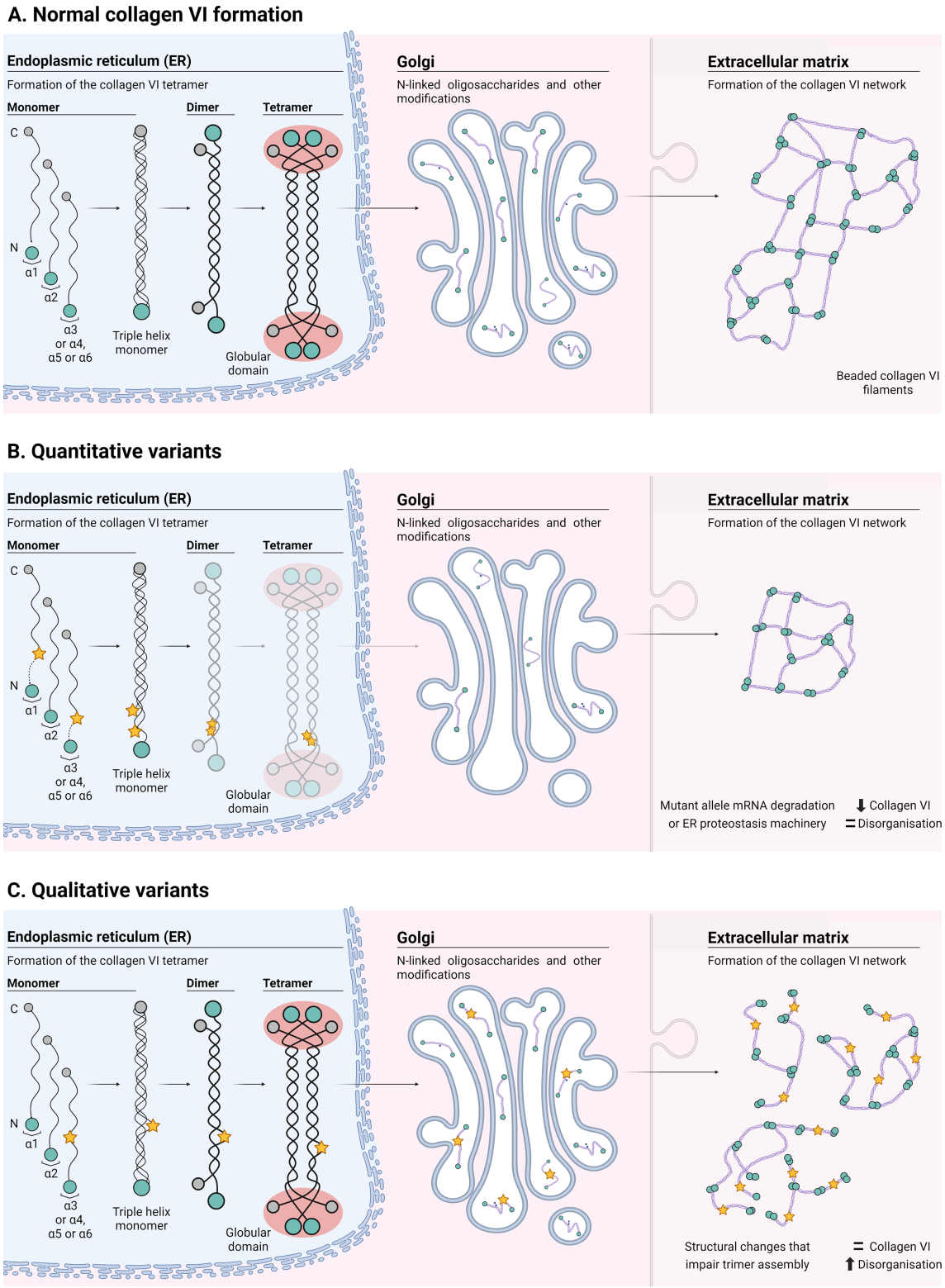

FIGURE 1 | **(A)** Normal Collagen VI assembly and secretion. **(B)** In the case of quantitative variants, such as nonsense variants, a second variant is required to be disease causing. Mutant allele mRNA is commonly completely degraded; if the degradation is only partial, some mutant truncated collagen chains may be produced, but they may engage the endoplasmic reticulum (ER) proteostasis machinery. This phenomenon leads to a reduction in the amount of collagen VI in the ECM while ensuring the correct organisation of the network. **(C)** Qualitative variants, such as missense variants, have been shown to induce structural changes that impair trimer assembly or helix formation and structure. This, in turn, can result in increased matrix disorganisation without changing collagen VI levels.

TABLE 1 | Skin-derived fibroblast cultures used in this study.

Sample culture	Variant	Clinical significance	Inheritance	Variant effect	Severity	Doubling time (days)	Origin
Controls							
CTRL-1	—	—	—	—	—	2.1	Biogipuzkoa
CTRL-2	—	—	—	—	—	2.3	Lonza
CTRL-3	—	—	—	—	—	1.9	BHISJDI
CTRL-4	—	—	—	—	—	3.1	BHISJDI
Patients							
COLVI-1	*COL6A1*: het. c.1056 + 1 G > A	Pathogenic	AD	Exon 14 skipping	BM	2.6	BHISJDI
COLVI-2	*COL6A1:* het. c.877 G > A	Pathogenic	AD	Missense (Gly > Arg)	Intermediate COL6-RD	2.0	BHISJDI
COLVI-2edit	CR RNA2[a]	—	—	—	—	3.2	BHISJDI
COLVI-3	*COL6A2:* het. c.2329 T > C	Likely pathogenic	AR	Missense (Cys > Arg)	UCMD	8.6	BHISJDI
	COL6A2: het. c.403 G > A	Likely pathogenic	AR	Missense (Asp > Asn)			
COLVI-4	*COL6A2:* hom. c.1970-9 G > A	Pathogenic	AR	Nonsense mutation	UCMD	1.9	BHISJDI
COLVI-5	*COL6A2:* het. c.1970-9 G > A	Pathogenic	AR	Nonsense mutation	UCMD	1.5	BHISJDI
	COL6A2: het. c.115 + 2 T > C	Pathogenic	AD	Nonsense mutation			

[a]CR RNA2-COLVI-2 cells edited with CRISPR/Cas9 RNA guide 2 (crRNA2) by Dr. López-Márquez in Sant Joan de Déu [17].

catalogue number CC-2511, Lonza Bioscience, Morrisville, NC, USA), obtained from Biogipuzkoa Health Research Institute (Donostia-San Sebastian, Spain) or sourced from the Biobank of the Hospital Sant Joan de Déu (BHISJDI, Barcelona, Spain). The previously generated edited culture pair (COLVI-2 and COLVI-2edit), derived from a COL6-RD patient with an intermediate phenotype, was provided by Drs. Jiménez-Mallebrera and López-Márquez [17].

2.2 | Cell Culture

Fibroblasts were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% Foetal Bovine Serum (FBS), 2% GlutaMax and 1% Penicillin–Streptomycin-Glutamine (all from Gibco, Waltham, MA, US) and tested for mycoplasma contamination using VenorGeM Classic kit (Minerva BioLabs, Berlin, Germany). Cells were cultured at 37°C in 5% CO_2.

2.3 | Immunocytochemistry Procedure

Skin fibroblasts were seeded at a density of 1×10^4-2×10^4 cells per well on 24-well plates containing glass coverslips that had been pre-coated with collagen I (Sigma-Aldrich, St. Louis, MO, US) in accordance with the manufacturer's instructions. The growth medium was then replaced with a medium enriched with ascorbic acid (L-ascorbic acid phosphate 2-magnesium, Sigma-Aldrich, St. Louis, MO, US) according to the experimental design. Following the designated incubation time in ascorbic acid, cells were rinsed with PBS (Gibco, Waltham, MA, US) and fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, US) for 10 min, followed by four 5-min washes in PBS.

For visualising both intracellular and extracellular collagen VI, cells in some wells were permeabilised by washing them four times with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, US) in PBS for 5 min. To observe only the extracellular collagen VI, the wells of interest were washed four times with PBS alone. Subsequently, all samples were then blocked with Intercept (PBS) blocking buffer (LI-COR Biosciences, Lincoln, NE, US) for 2 h and immunolabelled with anti-collagen VI antibody (Merck Millipore, Burlington, MA, US, MAB1944, 1:2500) diluted in blocking buffer or with Cy3 conjugated Collagen-Hybridising Peptide (CHP Cy3 conjugated, 3Helix, Utah, US) diluted in PBS for 1 h. As specified in the manufacturer's indications, CHP should be heated at 80°C for 10 min and immediately placed on ice for another 10 min before use. After being washed four times in PBS, cells were blocked for 5 min with blocking buffer, followed by a 1-h incubation with an

appropriate secondary antibody (diluted 1:500 in blocking buffer), while being protected from light.

The cells were then subjected to a 10-min wash with 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO, US) in PBS, incubated in Hoechst 33342 solution (Thermo Fisher Scientific, Waltham, MA, US) diluted 1:1000 in PBS for 5 min, washed three times with 0.05% Tween-20 in PBS and once in PBS. Finally, slides were mounted using ProLong antifade mounting medium (Thermo Fisher Scientific, Waltham, MA, US) and kept at 4°C. Images were captured using a Leica DMIL-LED epifluorescence microscope with a 10x objective and LAS X software (Leica Microsystems, Wetzlar, Germany).

2.4 | Flow Cytometry

Assays were performed according to the latest version of the protocol proposed by Kim et al. [13], with some modifications. Skin fibroblasts were seeded at a density of 1×10^6–2×10^6 cells in a 175 cm^2 tissue culture flask. The growth medium was replaced with a medium containing ascorbic acid 48 and 96 h after seeding, and the cells were further incubated for 72 h. Cells were then washed twice in PBS (Mg^{2+} and Ca^{2+} free, Gibco, Waltham, MA, US) and once in Cell Dissociation Buffer (enzyme free, PBS, Gibco, Waltham, MA, US). Cells were harvested with the Cell Dissociation Buffer, counted to obtain 0.5×10^6 cells/tube and fixed with 2% paraformaldehyde for 10 min on ice. The cells were then washed twice with PBS and centrifuged at 4°C for 5 min at 500 g. Pellets were re-suspended and incubated with either a monoclonal primary antibody against collagen type VI (Merck Millipore, Burlington, MA, US, MAB1944, 1:250) or with a monoclonal primary antibody against fibroblast surface protein (Sigma-Aldrich, St. Louis, MO, US, F4771, 1:20) in PBS/0.1% FBS or PBS/0.05% FBS and Tween-20 (for permeabilisation) for 30 min on ice. A negative control was set up incubating cells without the primary antibody on ice. Cells were then washed with PBS/0.1% FBS with or without Tween-20 and centrifuged at 500 g for 5 min at 4°C. Secondary antibodies (Alexa Fluor 488 goat anti-mouse IgM and Alexa Fluor donkey anti-mouse IgG1, Invitrogen Life Technologies, Carlsbad, CA, US) were diluted (1:500) with either PBS/0.1% FBS or PBS/0.05% FBS with Tween 20 and incubated for 20 min on ice. Subsequently, cells were washed with PBS, centrifuged at 500 g for 5 min at 4°C, resuspended in PBS and analysed using a MACSQuant X flow cytometer (MACSQuant: Miltenyi Biotec, Cologne, Germany).

2.5 | In Cell/On Cell Western Assay (Collablot)

Skin fibroblasts were seeded into collagen I-coated 96-well plates (Thermo Fisher Scientific, Waltham, MA, US) at a density designed to achieve roughly 1.6×10^4 cells per well after 72 h. Subsequently, the growth medium was replaced with L-ascorbic acid phosphate 2-magnesium (Sigma-Aldrich, St. Louis, MO, US) according to the experimental design. After the designated incubation period in the L-ascorbic acid phosphate 2-magnesium medium, cells were fixed in 4% paraformaldehyde for 10 min and washed four times for 5 min in PBS. Samples were then incubated with DNA stain DRAQ5 (Thermo Fisher Scientific, Waltham, MA, US) diluted 1:1000 in PBS for 1 h, after which the plates were scanned using an Odyssey M imaging system (Odyssey M: LI-COR Biosciences, Lincoln, NE, US). The cells were subsequently permeabilised and blocked as per immunocytochemistry sample preparation protocols and incubated overnight at 4°C with either an anti-collagen VI primary antibody or a Collagen-Hybridising Peptide (CHP, 3Helix, Utah, US) in a blocking buffer. As specified in the manufacturer's indications, CHP should be heated at 80°C for 10 min and immediately placed on ice for another 10 min before use. After incubation, cells were washed four times in PBS and incubated with the appropriate secondary antibodies (LI-COR Biosciences, Lincoln, NE, US) diluted at 1:800 in blocking buffer for 1 h. Cells were then washed three times in 0.05% Tween-20 in PBS, followed by two washes in PBS. The plates were scanned using an Odyssey M plate reader and data were analysed using Empiria Studio 2.3 software (LI-COR Biosciences, Lincoln, NE, US) (Figure 2A).

To ensure accurate quantification of both molecules, the analysis of CHP and collagen VI was conducted on separate wells. This approach was necessary due to the intercalating nature of CHP peptides, which may be hindered by antibodies binding to collagen. Wells stained to identify either collagen VI or CHP, which also include DRAQ5 for normalisation, are shown in green, wells in red indicate staining with DRAQ5 and the secondary antibodies and wells in blue indicate no primary antibody control and no DRAQ5 (Figure 2B). Water was added to the outer wells of all plates to prevent evaporation from the inner wells. The plate was divided such that Columns 2–6 were non-permeabilised to analyse extracellular collagen, while Columns 7–11 were permeabilised to analyse total collagen. All readings were taken from the same plate utilising an Odyssey scanner at different wavelengths: 800 CW for collagen VI, 520 CW for CHP and 700 CW for DRAQ5.

The data collected via the Odyssey scanner were analysed as follows: First, the average of the readings from the corresponding no-primary background control wells was subtracted from each sample value. Following that, this background-corrected collagen VI or CHP data was normalised by dividing it by the DRAQ5 value of the same well. ((Value of a well in the 800 CW or 520 CW channel minus average nonprimary control wells)/value of the well in the 700 CW channel).

2.6 | Statistical Analyses

Data were analysed using GraphPad Prism 10 (GraphPad Software Inc. La Jolla, CA, US). Normality tests were applied using different methods to test distributions, including D'Agostino and Pearson, Anderson–Darling, Shapiro–Wilk and Kolmogorov–Smirnov normality tests. Homoscedasticity was assessed with an *F*-test. Unpaired Student's *t*-tests were applied in the case of samples following a normal distribution and homoscedasticity, and the non-parametric Mann–Whitney test was applied in all other cases. Data are presented as mean ± SD Differences are reported as significant as $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) and $p<0.0001$ (****).

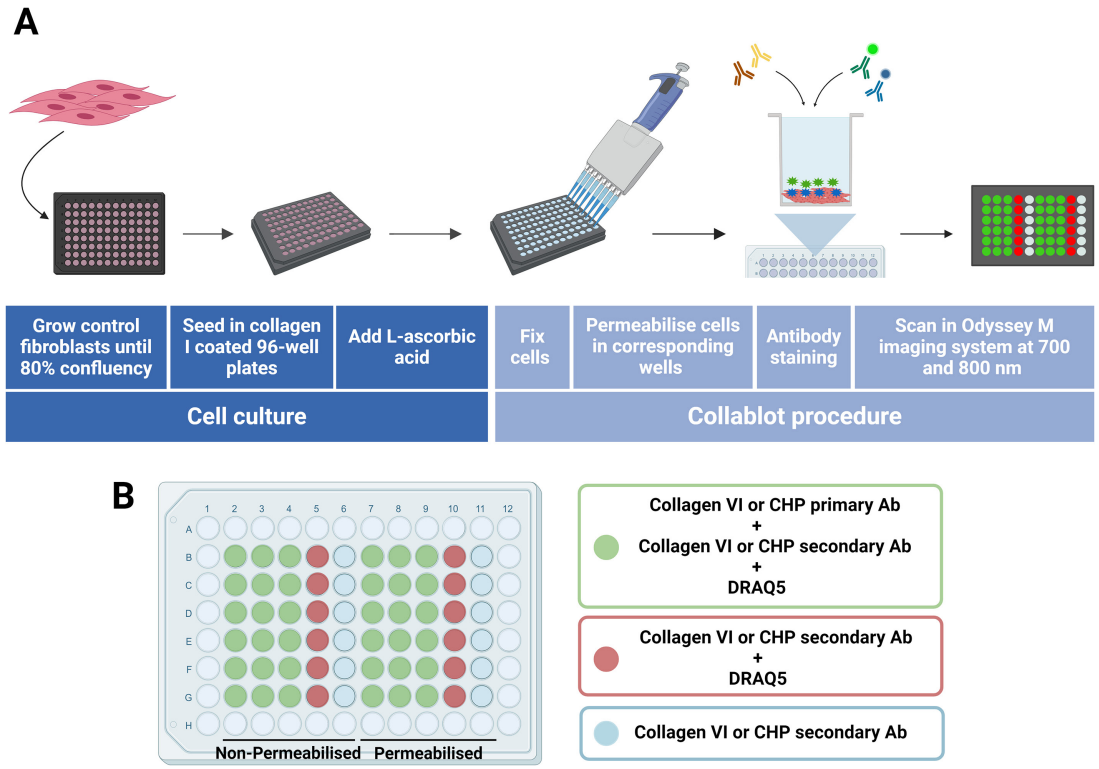

FIGURE 2 | **(A)** Illustration of collablot technique workflow. The first part of the workflow consists of cell culture and collagen secretion stimulation. The second is the collablot, where cells are fixed and incubated with a primary antibody, followed by its secondary antibody and then collagen or disorganisation is quantified. **(B)** Set up of the plates. Plates are divided into permeabilised and non-permeabilised. Wells in red indicate staining with DRAQ5, and wells in green indicate staining with collagen VI antibody or CHP.

3 | Results

3.1 | Collablot Optimisation

3.1.1 | Seeding Density/Duplication Times

Having a thorough understanding of cell cultures is essential when implementing any ICW technique, primarily due to the variability in doubling times among primary cultures. Table 1 summarises the doubling times of the cultures used in this study. When using cultures from different patients, adjustments must be made to account for the differences in doubling times, such as by varying the number of cells seeded per well.

To accurately quantify the DRAQ5 signal (700 CW), used to normalise each well for cell density, it must fall within a linear range. To establish the number of cells needed to be within this range, several cell dilutions were conducted. In these experiments, a control culture which displays intermediate growth characteristics, CTRL-1 (see Figure S1), was used. After studying this and other cultures, it was established that an initial seeding density of 6500 cells/well was appropriate for intermediate-growing cultures (CTRL-1, CTRL-2, CTRL-3, CTRL-4, COLVI-1 and COLVI-2edit), 5300 cells/well for fast-growing cultures (COLVI-2, COLVI-4, COLVI-5) and 12,000 cells/well for slow-growing cultures (COLVI-3).

3.1.2 | Selection of Primary Antibodies and Collagen-Hybridising Peptide

The basis of ICW and OCW assays is immunocytochemical, and a good selection of antibodies is necessary. The secondary antibodies and DRAQ5 were used at the concentrations suggested by each manufacturer. To select the most appropriate collagen VI primary antibody, a panel consisting of three different antibodies previously validated for immunofluorescence (see Table S1) was evaluated. The antibodies compared were an anti-human collagen VI polyclonal antibody produced in rabbit (Abcam, Cambridge, UK: ab6588) and two anti-human collagen VI antibodies produced in mice (Merck Millipore, Burlington, MA, US: MAB3303 and MAB1944) [7, 10, 12, 13, 18–27].

The ICW/OCW methodology detects native protein, as samples are not lysed or denatured. Therefore, it is advisable to evaluate the antibodies through a standard immunocytochemistry experiment. The CTRL-1 cell line was probed with the three primary antibodies (Table S1) and their respective secondary antibodies, Alexa Fluor 488 anti-rabbit IgG (Fisher Scientific, Hampton, NH, US) for ab6588 and Alexa Fluor 488 anti-mouse IgG (Fisher Scientific, Hampton, NH, US) for MAB3303 and MAB1944. As illustrated in Figure S2, while the anti-rabbit secondary antibody showed a non-specific signal in the absence of the primary antibody, the anti-mouse

secondary antibody showed no such non-specific signal, confirming its suitability for use. The polyclonal antibody ab6588 displayed minimal signal, and the faint signal detected could be attributed to the non-specific signal generated by the secondary antibody. In contrast, both monoclonal primary antibodies demonstrated a positive response, thus, warranting further evaluation (Figure 3A).

To ascertain the more suitable monoclonal antibody for this type of assay and its optimal titration, an ICW/OCW assay was conducted using the CTRL-1 cell line and various antibody dilutions (1:1000, 1:2000, 1:4000, 1:10000 and 1:250000). The MAB1944 antibody exhibited a higher signal compared to the MAB3303 antibody at all tested concentrations, corroborating the results obtained in the immunohistochemistry assay. Furthermore, including both permeabilised and non-permeabilised cells, the greatest signal-to-background ratio was achieved with the 1:4000 dilution (Figure 3B). Consequently, the primary antibody MAB1944 at a dilution of 1:4000 was selected.

In addition to the amount of collagen produced and its distribution inside or outside cells, the level of disorganisation in the collagen filaments might also give valuable insights about the

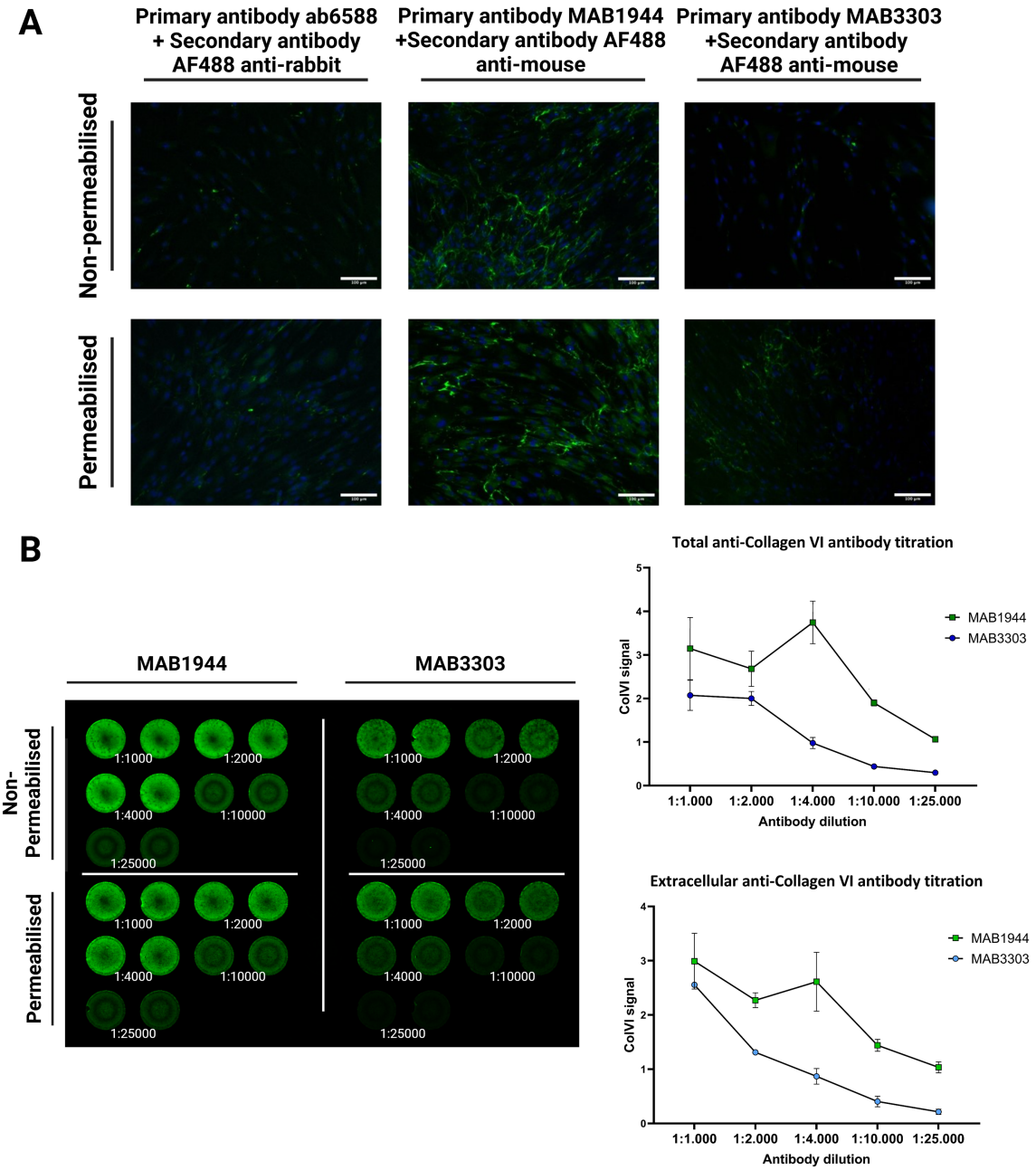

FIGURE 3 | **(A)** Immunocytochemical analysis of the three different primary antibodies for collagen VI (ab6588, MAB1944 and MAB3303; 1:2500) was carried out using the CTRL-1 line, permeabilised and non-permeabilised. A single replicate with two repeats. **(B)** Collablot analysis with two different primary antibodies for collagen VI (MAB1944 and MAB3303) titrations (1:1000, 1:2000, 1:4000, 1:10000 and 1:25000) carried out using CTRL-1 line, permeabilised (total collagen) and non-permeabilised (extracellular collagen). ColVI signal refers to the DRAQ5-adjusted ColVI signal. A single replicate with two repeats. Data was analysed using the non-parametric Mann–Whitney test.

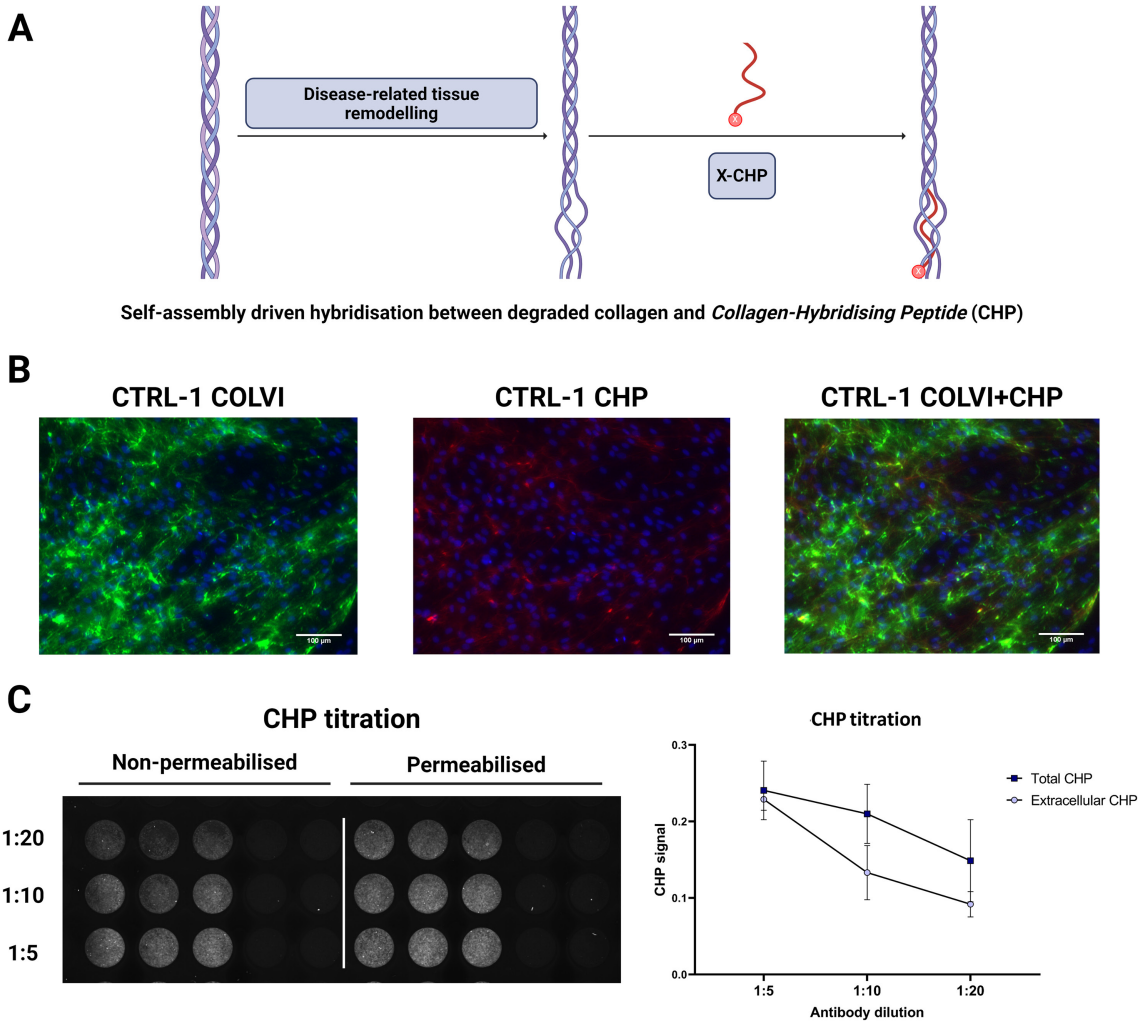

FIGURE 4 | **(A)** Mechanism of action of the Collagen-Hybridising Peptide (CHP). **(B)** Immunocytochemical analysis of the colocalisation of the CHP (1:5) and the collagen VI (1:2500) was carried out using a non-permeabilised CTRL-1 line. A single replicate with two repeats. **(C)** Collablot analysis of CHP titrations (1:5, 1:10 and 1:20) carried out using CTRL-1 line, permeabilised and non-permeabilised. CHP signal refers to the DRAQ5-adjusted CHP signal. A single replicate with three repeats. Data was analysed using a non-parametric Mann–Whitney test.

cultures. Therefore, a peptide capable of intercalating among loosely bound collagen fibres (CHP) was assessed (Figure 4A). To assess whether the CHP colocalised with collagen VI, an immunocytochemical analysis was conducted (Figure 4B). To determine the optimal titration of CHP to use, another ICW/OCW assay was conducted on the same control line, using different CHP dilutions (1:5, 1:10 and 1:20) (Figure 4C) selected taking into account the manufacturer's indications, where CHP titration should be between 5 and 30 μM. Considering both permeabilised and non-permeabilised cells, the highest signal-to-background ratio was achieved with the 1:10 dilution. Therefore, the CHP dilution of 1:10 was selected.

3.1.3 | Optimisation of L-Ascorbic Acid Concentration for Collagen VI Secretion

Ascorbic acid is essential for collagen VI synthesis and promotes the secretion of this protein into the extracellular medium [28]. An immunocytochemical assay (Figure S3) was performed to determine the best incubation time and concentration of ascorbic acid required to detect variations in collagen VI synthesis and secretion. This experiment confirmed that both ascorbic acid concentration and incubation time are crucial factors affecting collagen secretion: the higher these parameters, the greater the secretion. We were able to quantify this observation using the ICW/OCW (collablot) assay (Figure 5): with this technique, it was possible to visualise not only the optimal combination of a concentration of ascorbic acid of 50 μg/μL with a 48-h incubation, as described in other studies [6–8, 10, 29], but also demonstrated the collablot's ability to detect various concentrations of collagen both extracellularly and intracellularly.

3.2 | Collablot of Control and Patient Cultures

Cultures derived from control and patient biopsies were analysed using the immunocytochemical assay and our optimised collablot method (Figure 6 and Figure S4). The immunocytochemical assay (Figure 6A and Figure S4A) provides qualitative data, while the collablot quantifies extracellular and total collagen (Figures 6B–D and Figure S4B) and matrix disorganisation (Figure 6E and Figure S4C). The quantification of collagen with

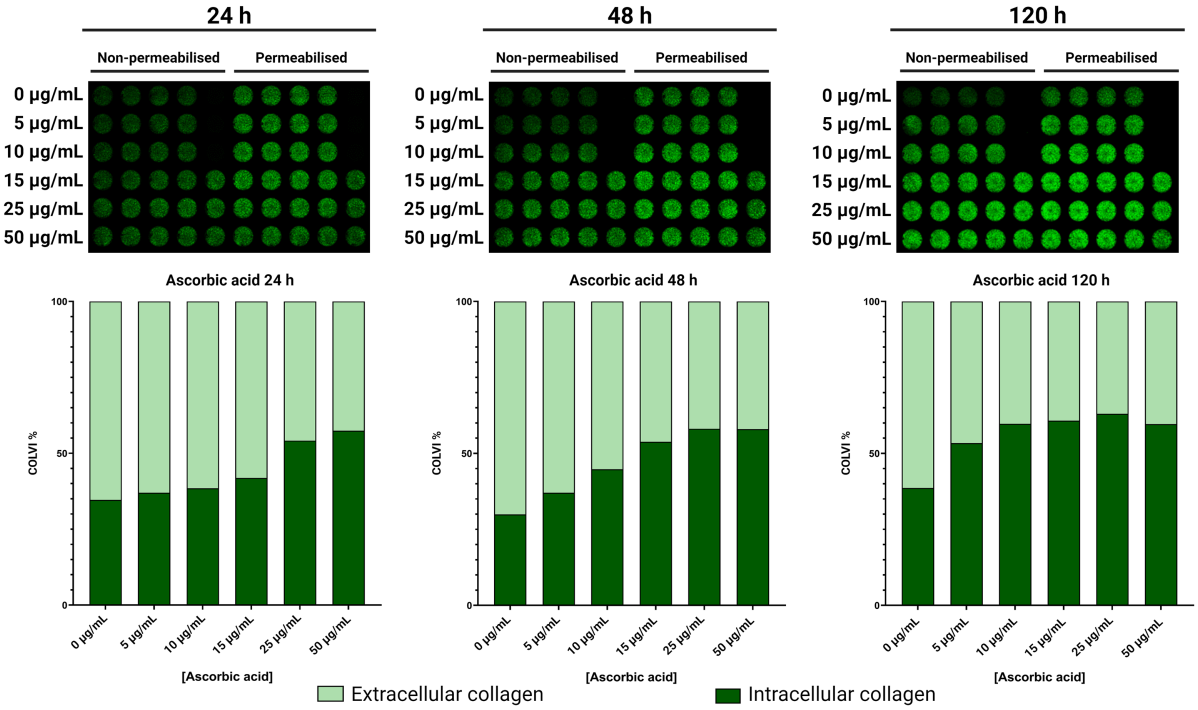

FIGURE 5 | Collablot analysis with the same parameters as the ICC and the quantitative results of both intracellular and extracellular collagen VI expressed due to the effect of ascorbic acid. Carried out using permeabilised and non-permeabilised CTRL-1 line. A single replicate with four repeats.

the collablot mirrored the immunocytochemistry results: while a trend for lower collagen content and a larger percentage of intracellular collagen can be detected, it is not possible to statistically distinguish all the patients from control groups due to the heterogeneity present among patients, highlighting the need for additional analysis.

The statistical analysis of total collagen (see Figure 6D) revealed that, except for patient COLVI-3 (who has a qualitative missense variant), all patients showed a significant decline in total collagen, with patient COLVI-1 (who has a qualitative exon 14 skipping variant) showing the least significant decline. When we analysed total collagen into extracellular collagen on one side (Figure 6B) and intracellular collagen on the other (Figure 6C), we saw that the decrease in total collagen was mainly due to the drop in extracellular collagen. Only one patient, COLVI-4, with a quantitative nonsense variant, showed a significant decrease in intracellular collagen. In contrast, all patients except COLVI-3 exhibited a decrease in extracellular collagen.

The quantification by collablot of the CHP peptide signal in these cultures was indicative of the levels of organisation of the extracellular collagen matrix and showed that the levels of CHP were diverse among the different cell lines tested. As this signal is also dependent on the extracellular amount of collagen, it was shown relative to the extracellular collagen amount present in each culture (Figure 6E). It should be noted that the results presented in the CHP immunocytochemistry experiment (Figure S4A) do not directly correspond to the CHP/ColVI ratio, as they merely reflect extracellular CHP. When the data was displayed in this way, control cultures presented a lower CHP/ColVI ratio when compared to patient cultures, corresponding with the amount of peptide being able to intercalate within the fibres due to disorganisation.

A detailed analysis of the cell lines showed that patients with qualitative variants, such as COLVI-2 and COLVI-3 (both missense variants), had the highest CHP/ColVI ratio compared to the mean average of controls and therefore, the highest significance. However, patient COLVI-1, with an exon 14 skipping variant, which is also considered a qualitative variant, had a lower significance compared to missense variants. Patients with quantitative variants, such as COLVI-4 and COLVI-5 (both nonsense variants), had the lowest CHP/ColVI ratio compared to the mean of controls, with low or no significance, respectively.

3.3 | Evaluation of Collagen VI Therapeutic Restoration by Collablot

As one of the objectives of this study was to develop a method that could be used to screen new treatments, we used it to evaluate an isogenic cell culture pair: a 'rescued' culture was created after gene editing of a patient's fibroblast culture, and these cultures were kindly provided by HSJD and analysed in Biobizkaia. The cultures had been characterised in the original publication [17], and we confirmed that characterisation by subjecting the cultures to analysis by immunocytochemistry (Figure 7A), collagen collablot (Figure 7B), flow cytometry (Figure 7C) and CHP analysis by collablot (Figure 7D).

It was possible to corroborate the restoration of collagen expression described in the original publication by immunohistochemistry and collagen collablot. With our technique, it was confirmed that both total and extracellular collagen signals

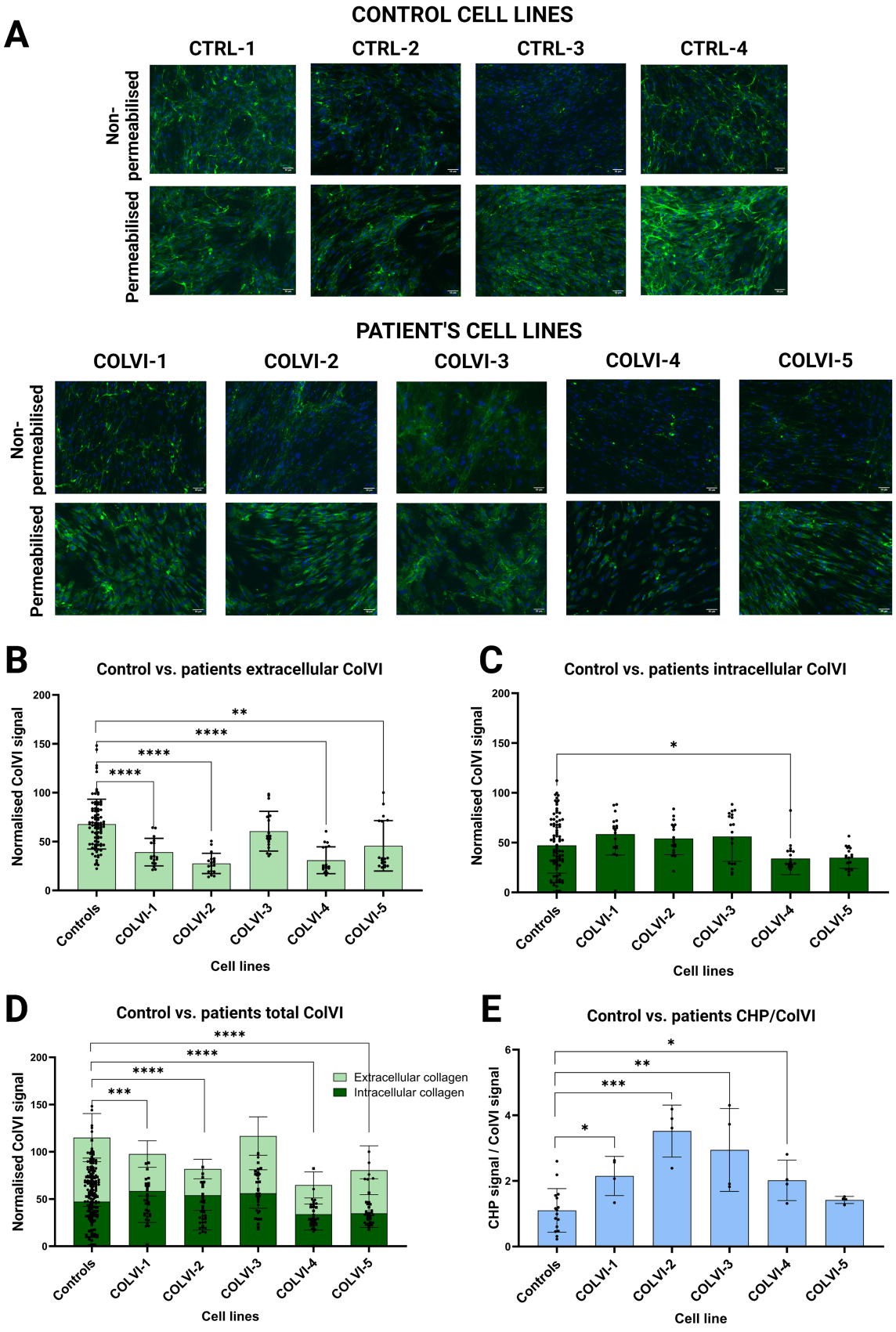

FIGURE 6 | Legend on next page.

FIGURE 6 | **(A)** Immunocytochemical results of collagen VI for each cell line. Two replicates with two repeats per replicate. **(B)** Statistical analysis of extracellular collagen from each patient compared to the control media (CTRL-1, CTRL-2, CTRL-3 and CTRL-4). Six replicates with six repeats per replicate. Data was analysed using a non-parametric Mann–Whitney test. **(C)** Statistical analysis of intracellular collagen from each patient compared to the control media (CTRL-1, CTRL-2, CTRL-3 and CTRL-4). Six replicates with six repeats per replicate. Data was analysed using a non-parametric Mann–Whitney test. **(D)** Collablot results showing intracellular and extracellular collagen VI of the control media (CTRL-1, CTRL-2, CTRL-3 and CTRL-4) and every patient. Statistical analysis of total (extracellular plus intracellular) collagen from each patient compared to the control media (CTRL-1, CTRL-2, CTRL-3 and CTRL-4). Six replicates with six repeats per replicate. Data was analysed using a non-parametric Mann–Whitney test. **(E)** Extracellular CHP signal normalised by extracellular ColVI signal obtained from on-cell western experiments of all cell lines. Four replicates with three repeats per replicate. Data was analysed using a non-parametric Mann–Whitney test.

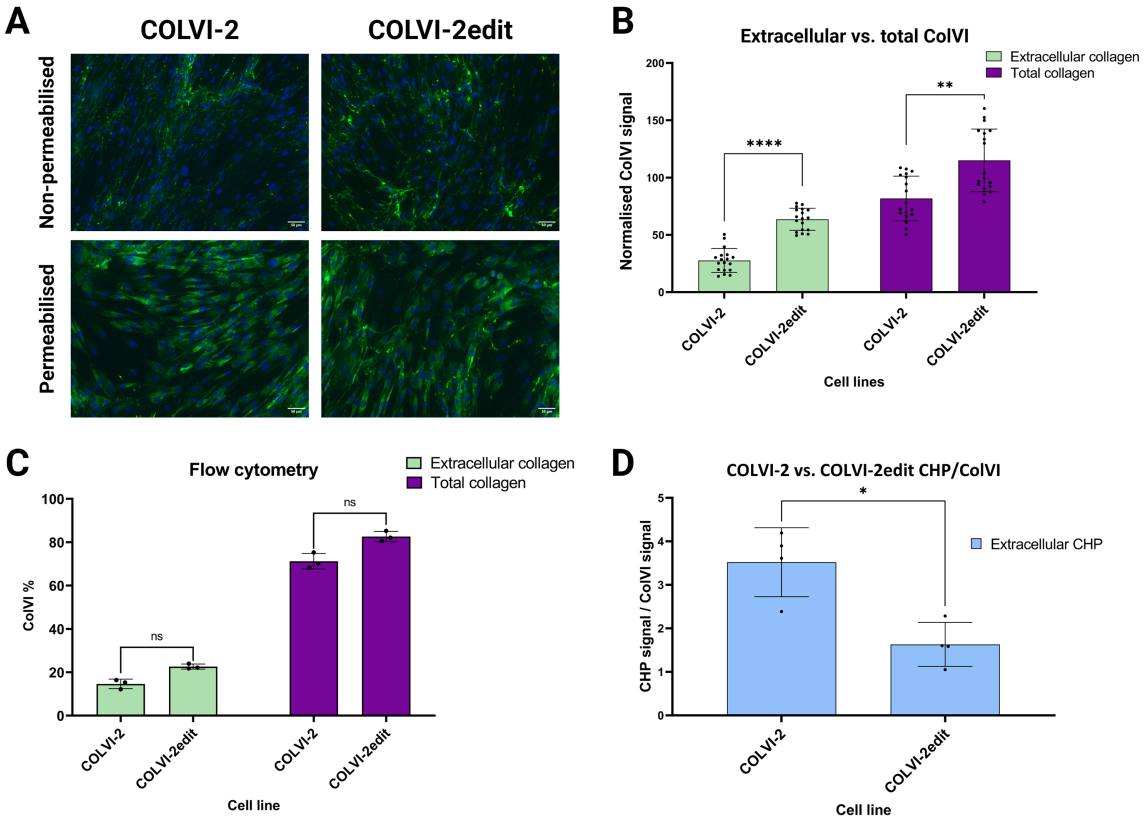

FIGURE 7 | **(A)** Immunocytochemical results for COLVI-2 and its edited line, COLVI-2edit, extracellular and total collagen (extracellular plus intracellular collagen). **(B)** Collablot results of COLVI-2 cell line and its edited line, COLVI-2edit. Six replicates with three repeats per replicate. Data was analysed using the parametric t-test in the case of extracellular collagen and the non-parametric Mann–Whitney test in the case of total collagen. **(C)** Flow cytometry results of COLVI-2 cell line and its edited line, COLVI-2edit. A single replicate with three repeats. Data was analysed using the non-parametric Mann–Whitney test. **(D)** Extracellular CHP signal normalised by extracellular ColVI signal of COLVI-2 cell line and its edited line, COLVI-2edit. Four replicates with three repeats per replicate. Data was analysed using the non-parametric Mann–Whitney test.

were higher in the edited cultures. Additionally, CHP quantification by collablot confirmed a very significant decrease in the extracellular fraction, corresponding to a lower amount of CHP bound to denatured collagen strands, suggesting that after the edition of the COLVI-2 cell line, the collagen matrix is more organised and structured. However, analysis via flow cytometry revealed no significant increase in either the extracellular or intracellular collagen-secreting cell percentage post-therapy.

4 | Discussion

The diagnosis of myopathies associated with collagen deficiency requires, in addition to clinical assessment, confirmation of collagen deficiency as a complementary assay to genetic analysis of the *COL6A1*, *COL6A2* and *COL6A3* genes. This is a problem because most diagnostic techniques are not quantitative, and those that are do not accurately distinguish patients with the milder versions of the disease, as their collagen levels are indistinguishable from controls. The only quantitative method capable of distinguishing BM from UCMD reported so far is flow cytometry. However, this is an indirect quantification, as it measures the number of collagen-expressing cells, not the amount of collagen excreted [13].

The structural and organisational consequences of *COL6* variants depend on their location. Those at the N-terminal of the triple helix domain always impair the assembly of tetramers and microfibrils. However, for variants located outside the triple helix domain, the disorganisation of the collagen network depends on whether they

prevent incorporation into monomers and are then targeted for proteasomal degradation, or whether they are incorporated into monomers, dimers and/or tetramers that disrupt the formation and/or organisation of microfibrils [5, 30].

We have now developed a method that may contribute to the faster development of new treatments for neuromuscular disorders, as it allows more accurate quantification of collagen VI with the use of a limited number of cells. Unfortunately, collagen quantification alone would not suffice as a diagnostic tool because there are patients who express equal or greater amounts of both extracellular and intracellular collagen compared to some of the controls. This suggests that the disease is not solely determined by the quantity of collagen but also that the organisation of collagen plays a significant role in the disease. Some studies [31–34] have demonstrated the ability of the collagen-hybridising peptide (CHP) to bind unfolded collagen chains and thus show damage at the molecular level. The incorporation of this molecule into our technique has demonstrated a large disruption of the collagen network in patients relative to the control cohort. While CHP quantification is not sufficient for a definitive diagnosis of COL6-RD, in combination with the quantification of collagen expression, it provides valuable insight into the tissue damage present in the patients under study.

We have demonstrated that collablots can detect increased matrix disorganisation in the case of qualitative variants (COLVI-1, COLVI-2 and COLVI-3 patients) and reduced collagen quantity, both extracellular and intracellular and total, in the case of quantitative variants (COLVI-4 and COLVI-5 patients). However, in certain cases, qualitative variants have also been observed to result in a substantial decrease in collagen levels (as evidenced in COLVI-1 and COLVI-2 patients). This phenomenon can be attributed to the activation of degradation pathways, such as the ER-associated degradation (ERAD) pathway or the cytotoxic unfolded protein response (UPR), which are implicated in the degradation of collagen [35, 36]. Furthermore, quantitative variants are generally linked to a decline in collagen production without an increase in disorganisation [37, 38] (see patient COLVI-5). However, incomplete degradation of mRNA or truncated proteins can increase disorganisation of the extracellular matrix (see patient COLVI-4), which contradicts the expected outcomes of such variants [5].

Even though the collablot method is a robust tool that provides quantification of both collagen VI and the organisation levels of the extracellular collagen, it is likely to be insufficient for the diagnosis of this disorder. The myopathies caused by collagen deficiencies are complex conditions, and several other factors could influence their diagnosis. One key factor is the penetrance of the variants, the large number of variants of unknown meaning and the frequent mosaicism found in this type of disease [1].

Nevertheless, this method is a quantifiable and useful method for the evaluation of novel therapies, as demonstrated in this manuscript with the characterisation of a cell culture isogenic pair: one including a variant in the *COL6A1* gene and an edited version created by CRISPR/Cas9 [17]. The method we have described easily showed how collagen VI levels were increased in the edited cell line compared to the original cell line from the patient. Remarkably, the analysis of CHP levels showed a very marked improvement in the organisation of collagen VI in the extracellular matrix of the edited cell line. As extracellular matrix disorganisation is a key characteristic of this disease, we want to highlight the suitability of our method for evaluating this feature.

Our results show that collablots could be a fast and suitable technique for the quantification of both collagen VI and its disorganisation in skin-derived fibroblasts. Although this quantification is not sufficient for disease diagnosis on its own, it can provide new insights available to clinicians, and it is a suitable method for assessing *in vitro* the impact of therapies on collagen expression. We expect that this technique will be useful for the neuromuscular community.

Author Contributions

Conceptualisation, V.A.-G.; methodology, validation, formal analysis and investigation, resources, V.A.-G.; writing-original draft preparation, M. S.; N. O.-B.; writing-review and editing, A. L.-M.; C. J.-M.; A. L.-M.; M. S.; I. G.-M.; V. A.-G.; visualisation, supervision, A. L.-M. M. S.; I. G.-M.; V. A.-G.; project administration and funding acquisition, V. A.-G.

Acknowledgements

We strongly acknowledge Fundación Noelia for supporting this work and for its enormous effort in securing funding, their trust in our work, and their extraordinary cooperation.

We acknowledge the use of cell cultures provided by Dr López de Munain, Biogipuzkoa Health Research Institute (Donostia-San Sebastián, Spain), and the Biobank (BHISJDI) of the Hospital Sant Joan de Déu (HSJD) (Barcelona, Spain).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All relevant data can be found within the article and its supplementary information. All materials and further information on this study are available upon request.

References

1. A. R Foley, P. Mohassel, S. Donkervoort, V. Bolduc, and C. G. Bönnemann, "Collagen VI-Related Dystrophies," *GeneReviews®*. Published Online March 11, 2021, accessed July 24, 2024, https://www.ncbi.nlm.nih.gov/books/NBK1503/.

2. H. Von Der Mark, M. Aumailley, G. Wick, R. Fleischmajer, and R. Timpl, "Immunochemistry, Genuine Size and Tissue Localization of Collagen VI," *European Journal of Biochemistry* 142, no. 3 (1984): 493–502, https://doi.org/10.1111/j.1432-1033.1984.tb08313.x.

3. Y. Zou, R. Z. Zhang, P. Sabatelli, M. L. Chu, and C. G. Bönnemann, "Muscle Interstitial Fibroblasts Are the Main Source of Collagen VI Synthesis in Skeletal Muscle: Implications for Congenital Muscular Dystrophy Types Ullrich and Bethlem," *Journal of Neuropathology and Experimental Neurology* 67, no. 2 (2008): 144–154.

4. A. Di Martino, M. Cescon, C. D'Agostino, et al., "Collagen VI in the Musculoskeletal System," *International Journal of Molecular Sciences* 24, no. 6 (2023): 5095, https://doi.org/10.3390/ijms24065095.

5. J. F. Bateman, M. D. Shoulders, and S. R. Lamandé, "Collagen Misfolding Mutations: The Contribution of the Unfolded Protein Response to the Molecular Pathology," *Connective Tissue Research* 63, no. 3 (2022): 210–227, https://doi.org/10.1080/03008207.2022.2036735.

6. E. Marrosu, P. Ala, F. Muntoni, and H. Zhou, "Gapmer Antisense Oligonucleotides Suppress the Mutant Allele of *COL6A3* and Restore Functional Protein in Ullrich Muscular Dystrophy," *Molecular Therapy - Nucleic Acids* 8 (2017): 416–427, https://doi.org/10.1016/j.omtn.2017.07.006.

7. S. Aguti, V. Bolduc, P. Ala, et al., "Exon-Skipping Oligonucleotides Restore Functional Collagen VI by Correcting a Common *COL6A1* Mutation in Ullrich CMD," *Molecular Therapy - Nucleic Acids* 21 (2020): 205–216, https://doi.org/10.1016/j.omtn.2020.05.029.

8. A. Brull, A. Sarathy, V. Bolduc, G. S. Chen, R. M. McCarty, and C. G. Bönnemann, "Optimized Allele-Specific Silencing of the Dominant-Negative *COL6A1* G293R Substitution Causing Collagen VI-Related Dystrophy," *Molecular Therapy - Nucleic Acids* 35, no. 2 (2024): 102178, https://doi.org/10.1016/j.omtn.2024.102178.

9. V. Bolduc, A. Reghan Foley, H. Solomon-Degefa, et al., "A Recurrent *COL6A1* Pseudoexon Insertion Causes Muscular Dystrophy and Is Effectively Targeted by Splice-Correction Therapies," *JCI Insight* 4, no. 6 (2019): e124403, https://doi.org/10.1172/jci.insight.124403.

10. C. Jimenez-Mallebrera, M. A. Maioli, J. Kim, et al., "A Comparative Analysis of Collagen VI Production in Muscle, Skin and Fibroblasts From 14 Ullrich Congenital Muscular Dystrophy Patients With Dominant and Recessive *COL6A* Mutations," *Neuromuscular Disorders* 16, no. 9–10 (2006): 571–582, https://doi.org/10.1016/j.nmd.2006.07.015.

11. V. Allamand, L. Merlini, and K. Bushby, "166th ENMC International Workshop on Collagen Type VI-Related Myopathies, 22–24 May 2009, Naarden, The Netherlands," *Neuromuscular Disorders* 20, no. 5 (2010): 346–354, https://doi.org/10.1016/j.nmd.2010.02.012.

12. D. Hicks, A. Lampe, R. Barresi, et al., "A Refined Diagnostic Algorithm for Bethlem Myopathy," *Neurology* 70, no. 14 (2008): 1192–1199.

13. J. Kim, C. Jimenez-Mallebrera, A. R. Foley, et al., "Flow Cytometry Analysis: A Quantitative Method for Collagen VI Deficiency Screening," *Neuromuscular Disorders* 22, no. 2 (2012): 139–148, https://doi.org/10.1016/j.nmd.2011.08.006.

14. E. Ruiz-Del-Yerro, I. Garcia-Jimenez, K. Mamchaoui, and V. Arechavala-Gomeza, "Myoblots: Dystrophin Quantification by in-Cell Western Assay for a Streamlined Development of Duchenne Muscular Dystrophy (DMD) Treatments," *Neuropathology and Applied Neurobiology* 44, no. 5 (2018): 463–473, https://doi.org/10.1111/nan.12448.

15. A. López-Martínez, P. Soblechero-Martín, C. Cataldi, et al., "VP.88 Characterisation of Cell Culture Models of Myotonic Dystrophy Type I by in-Cell Western Technology and Digital Droplet PCR," *Neuromuscular Disorders* 32, no. 1 (2022): S131–S132, https://doi.org/10.1016/j.nmd.2022.07.373.

16. Y. Li, C. A. Foss, D. D. Summerfield, et al., "Targeting Collagen Strands by Photo-Triggered Triple-Helix Hybridization," *Proceedings of the National Academy of Sciences of the United States of America* 109, no. 37 (2012): 14767–14772, https://doi.org/10.1073/pnas.1209721109.

17. A. López-Márquez, M. Morín, S. Fernández-Peñalver, et al., "CRISPR/Cas9-Mediated Allele-Specific Disruption of a Dominant *COL6A1* Pathogenic Variant Improves Collagen VI Network in Patient Fibroblasts," *International Journal of Molecular Sciences* 23, no. 8 (2022): 4410–4426, https://doi.org/10.3390/ijms23084410.

18. S. N. Bardakov, R. V. Deev, R. M. Magomedova, et al., "Intrafamilial Phenotypic Variability of Collagen VI-Related Myopathy Due to a New Mutation in the *COL6A1* Gene," *Journal of Neuromuscular Diseases* 8, no. 2 (2021): 273–285, https://doi.org/10.3233/JND-200476.

19. J. Brands, F. Steffen, J. Spennes, T. Leeb, and T. Bilzer, "*COL6A1* Related Muscular Dystrophy in Landseer Dogs: A Canine Model for Ullrich Congenital Muscular Dystrophy," *Muscle & Nerve* 63, no. 4 (2021): 608–616, https://doi.org/10.1002/mus.27162.

20. V. Bolduc, K. M. Minor, Y. Hu, et al., "Pathogenic Variants in *COL6A3* Cause Ullrich-Like Congenital Muscular Dystrophy in Young Labrador Retriever Dogs," *Neuromuscular Disorders* 30, no. 5 (2020): 360–367, https://doi.org/10.1016/j.nmd.2020.03.005.

21. R. N. Villar-Quiles, S. Donkervoort, A. De Becdelièvre, et al., "Clinical and Molecular Spectrum Associated With *COL6A3* c.7447A>G p.(Lys2483Glu) Variant: Elucidating Its Role in Collagen VI-Related Myopathies," *Journal of Neuromuscular Diseases* 8, no. 4 (2021): 633–645, https://doi.org/10.3233/JND-200577.

22. E. Demir, A. Ferreiro, P. Sabatelli, et al., "Collagen VI Status and Clinical Severity in Ullrich Congenital Muscular Dystrophy: Phenotype Analysis of 11 Families Linked to the *COL6* Loci," *Neuropediatrics* 35, no. 2 (2004): 103–112, https://doi.org/10.1055/s-2004-815832.

23. M. Tétreault, A. Duquette, I. Thiffault, et al., "A New Form of Congenital Muscular Dystrophy With Joint Hyperlaxity Maps to 3p23-21," *Brain* 129, no. 8 (2006): 2077–2084, https://doi.org/10.1093/brain/awl146.

24. O. C. Vanegas, R. Z. Zhang, P. Sabatelli, et al., "Novel *COL6A1* Splicing Mutation in a Family Affected by Mild Bethlem Myopathy," *Muscle & Nerve* 25, no. 4 (2002): 513–519, https://doi.org/10.1002/mus.10100.

25. A. K. Lampe, Y. Zou, D. Sudano, et al., "Exon Skipping Mutations in Collagen VI Are Common and Are Predictive for Severity and Inheritance," *Human Mutation* 29, no. 6 (2008): 809–822, https://doi.org/10.1002/humu.20704.

26. E. Martoni, S. Petrini, C. Trabanelli, et al., "Characterization of a Rare Case of Ullrich Congenital Muscular Dystrophy Due to Truncating Mutations Within the COL6A1 Gene C-Terminal Domain: A Case Report," *BMC Medical Genetics* 14, no. 1 (2013): 59, https://doi.org/10.1186/1471-2350-14-59.

27. V. Bolduc, Y. Zou, D. Ko, and C. G. Bönnemann, "SiRNA-Mediated Allele-Specific Silencing of a *COL6A3* Mutation in a Cellular Model of Dominant Ullrich Muscular Dystrophy," *Molecular Therapy - Nucleic Acids* 3 (2014): e147, https://doi.org/10.1038/mtna.2013.74.

28. S. Murad, D. Grove, K. A. Lindberg, G. Reynolds, A. Sivarajah, and S. R. Pinnell, "Regulation of Collagen Synthesis by Ascorbic Acid," *Proceedings of the National Academy of Sciences of the United States of America* 78, no. 5 (1981): 2879–2882, https://doi.org/10.1073/PNAS.78.5.2879.

29. M. Schnoor, P. Cullen, J. Lorkowski, et al., "Production of Type VI Collagen by Human Macrophages: A New Dimension in Macrophage Functional Heterogeneity," *Journal of Immunology* 180, no. 8 (2008): 5707–5719, https://doi.org/10.4049/JIMMUNOL.180.8.5707.

30. A. K. Kwong, Y. Zhang, R. S. Ho, et al., "Collagen VI-Related Myopathies: Clinical Variability, Phenotype-Genotype Correlation and Exploratory Transcriptome Study," *Neuromuscular Disorders* 33, no. 5 (2023): 371–381, https://doi.org/10.1016/j.nmd.2023.03.003.

31. J. Hwang, Y. Huang, T. J. Burwell, et al., "In Situ Imaging of Tissue Remodeling With Collagen Hybridizing Peptides," *ACS Nano* 11, no. 10 (2017): 9825–9835, https://doi.org/10.1021/acsnano.7b03150.

32. L. L. Bennink, Y. Li, B. Kim, et al., "Visualizing Collagen Proteolysis by Peptide Hybridization: From 3D Cell Culture to *In Vivo* Imaging," *Biomaterials* 183 (2018): 67–76, https://doi.org/10.1016/J.BIOMATERIALS.2018.08.039.

33. J. L. Zitnay, Y. Li, Z. Qin, et al., "Molecular Level Detection and Localization of Mechanical Damage in Collagen Enabled by Collagen Hybridizing Peptides," *Nature Communications* 8, no. 1 (2017): 14913, https://doi.org/10.1038/ncomms14913.

34. A. H. Lin, J. L. Zitnay, Y. Li, S. M. Yu, and J. A. Weiss, "Microplate Assay for Denatured Collagen Using Collagen Hybridizing Peptides,"

Journal of Orthopaedic Research 37, no. 2 (2019): 431–438, https://doi.org/10.1002/jor.24185.

35. S. Ito and K. Nagata, "Quality Control of Procollagen in Cells," *Annual Review of Biochemistry* 90 (2021): 631–658, https://doi.org/10.1146/annurev-biochem-013118.

36. R. P. Boot-Handford and M. D. Briggs, "The Unfolded Protein Response and Its Relevance to Connective Tissue Diseases," *Cell and Tissue Research* 339, no. 1 (2009): 197–211, https://doi.org/10.1007/S00441-009-0877-8.

37. R. J. Butterfield, A. R. Foley, J. Dastgir, et al., "Position of Glycine Substitutions in the Triple Helix of *COL6A1*, *COL6A2*, and *COL6A3* Is Correlated With Severity and Mode of Inheritance in Collagen VI Myopathies," *Human Mutation* 34, no. 11 (2013): 1558–1567, https://doi.org/10.1002/HUMU.22429.

38. S. R. Lamandé, M. Mörgelin, C. Selan, G. Joost Jöbsis, F. Baas, and J. F. Bateman, "Kinked Collagen VI Tetramers and Reduced Microfibril Formation as a Result of Bethlem Myopathy and Introduced Triple Helical Glycine Mutations," *Journal of Biological Chemistry* 277, no. 3 (2002): 1949–1956, https://doi.org/10.1074/JBC.M109932200.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.