Collagen VI-Related Myopathies:

Navigating through the Molecular Maze, Myomatrix and Clinical Manifestations on a Journey toward Clinical Trials

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I, Aileen Reghan Foley, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

ABSTRACT

The congenital muscular dystrophies are a clinically and genetically heterogeneous group of disorders characterised by a congenital onset of weakness and hypotonia, typically associated with dystrophic-appearing muscle biopsy findings. The spectrum of clinical phenotypes associated with the congenital muscular dystrophy subgroup resulting from a deficiency of collagen VI in the extracellular matrix of muscle are collectively termed 'collagen VI-related myopathies' and include the early onset Ullrich congenital muscular dystrophy and the milder and later onset Bethlem myopathy as well as a phenotype of intermediate severity called 'intermediate collagen VI-related myopathy.' A major goal of this research has been to study the natural history of respiratory insufficiency in the collagen VI-related myopathies by analyzing longitudinal forced vital capacity data in a large, international cohort. A total of 486 forced vital capacity measurements obtained in 145 genetically and/or biochemically confirmed collagen VI-related myopathy patients from 10 neuromuscular centres [United States (2), United Kingdom (2), Australia (2), Italy (2), France (1) and Belgium (1)] were analysed and the resulting clarification of the phenotypic stratification of collagen VI-related myopathies reported. Another focus of this research has been the evaluation and the refining of the challenging diagnostic pathway for collagen VI-related myopathy patients, including evaluations of the diagnostic role of muscle histopathology, skin fibroblast immunocytochemistry and flow cytometry studies as well as muscle ultrasound and muscle magnetic resonance imaging studies. Finally, this research has studied the role of next generation genetic sequencing technologies including whole-genome and exome sequencing in the assessment of patients evaluated for collagen VI-related myopathies and related conditions.

PUBLICATIONS

Jimenez-Mallebrera C, **Foley AR**, Bönnemann CG. Proteins of the Extracellular Matrix. In: Muscle Disease: Pathology and Genetics, 2nd Edition. Eds. Hans H. Goebel, Caroline A. Sewry and Roy O. Weller, International Society of Neuropathology. John Wiley & Sons, Ltd. 2013. 102-107. [*In press*]

Stevens E, Carss KJ, Cirak S, **Foley AR**, et al. Mutations in *B3GALNT2* cause congenital muscular dystrophy with hypoglycosylation of alpha-dystroglycan. Am J Hum Genet 2013;92(3):354-65.

Cirak S, **Foley AR**, Herrmann R, et al. *Isoprenoid synthase domain containing* gene mutations are a common cause of congenital and limb girdle muscular dystrophies. Brain 2013;136(Pt1):269-281.

Emmanuele V, Sotiriou E, Gutierrez Rios P, Ganesh J, Ichord R, **Foley AR**, Akman HO, DiMauro S. A novel mutation in the mitochondrial DNA cytochrome b gene (*MTCYB)* in a patient with mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes syndrome. J of Child Neurol 2013;28(2):236-242.

Collins J, **Foley AR**, Straub V, Bönnemann CG. Spontaneous keloid formation in patients with Bethlem myopathy. Neurology 2012;79(21):2158.

Kaufmann P, McDermott MP, Darras BT, Finkel RS, Sproule DM, Kang PB, Oskoui M, Constantinescu A, Gooch CL, **Foley AR**, et al. Prospective cohort study of Spinal Muscular Atrophy Types 2 and 3. Neurology 2012;79(18):1889-1897

Johnson JO, Gibbs JR, Megarbane A, Urtizberea JA, Hernandez DG, **Foley AR**, et al. Exome sequencing reveals riboflavin transporter mutations as a cause of motor neuron disease. Brain 2012;135(9):2875-82.

Pane M, Messina S, Vasco G, **Foley AR**, et al. Respiratory and cardiac function in congenital muscular dystrophies with alpha dystroglycan deficiency. Neuromuscul Disord 2012;22:685-689.

Kim J, Jimenez-Mallebrera C, **Foley AR**, et al. Flow cytometry analysis: A quantitative method for collagen VI deficiency screening. Neuromuscul Disord 2012;22:139-148.

Godfrey C, **Foley AR**, Clement E, Muntoni F. Dystroglycanopathies: coming into focus. Curr Opin Genet Dev 2011;21:278-285.

Kaufmann P, McDermott M, Darras B, Finkel R, Kang P, Oskoui M, Constantinescu A, Sproule D, **Foley AR**, et al. Observational study of spinal muscular atrophy type 2 and 3: functional outcomes over one year. Arch Neurol 2011;68:779-786.

Foley AR, Hu Y, Zou Y, et al. Large genomic deletions: a novel cause of Ullrich congenital muscular dystrophy. Ann Neurol 2011;69:206-211.

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Just over a century since Dr Frederick Eustace Batten first described the congenital muscular dystrophies while working at the Great Ormond Street Hospital for Children, I undertook this clinical research into a congenital muscular dystrophy subgroup, the collagen VI-related myopathies, while also working at the Great Ormond Street Hospital for Children.

I thank Professor Francesco Muntoni for giving me the tremendous opportunity to join his team of dedicated clinicians and researchers at the Dubowitz Neuromuscular Centre for 2 years. Thank you, Francesco, for your steadfast confidence in me and your guidance both clinically and academically, as my mentor and the primary supervisor of this research/degree. I hope we will be preparing for further clinical trials for collagen VI-related myopathy patients in the years ahead.

I am very grateful to Professor Caroline Sewry for serving as my secondary supervisor. Thank you, Caroline, for your helpful guidance, for sharing my great enthusiasm for muscle biopsy and for always answering my questions during muscle biopsy conference with a smile.

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neuromuscular disorders at the Children's Hospital of Philadelphia (USA) and for continuing to serve as a fantastic mentor -even from across the pond- during my time in London.

At the core of this research are the collagen VI-related myopathy patients, whose willingness to share their stories and participate in clinical research has made this work possible, and whose incredibly spirited personalities and *joie de vivre* have kept me inspired to keep fighting in their corner. You are the champions.

Most importantly, I would like to thank my family for their incredible support and understanding along my journey towards becoming a neuromuscular specialist- a journey which has taken me 'across the pond' and even on a 20 kilometre run to raise funds for the Muscular Dystrophy Campaign. (And to think that I had packed my ballet pointe shoes for my time in London but did not have a pair of running shoes!) I never could have run the Great North Run if it were not for a cause which is incredibly near and dear to my heart. I ran- and have undertaken this research- for my inspirational patients and for all individuals with neuromuscular disease. You have taught me that real strength is not something made in a gym but that which we draw upon in times of adversity.

I especially dedicate this work in memory of my dear friend, Tom Wilson, whose courage in the face of Duchenne muscular dystrophy has been a source of inspiration throughout my life. I know that it is more than mere coincidence that I have entered the field of neuromuscular disorders and now write this thesis from the very place you pursued your studies. I hope you can see that the fight to find treatments for muscular dystrophies goes on, ever inspired by the strength of spirit of those who have faced and continue to face these relentless conditions.

While I cannot say I ran the Great North Run 'really fast' as my nieces encouraged, I ran those 20 kilometres in the same way I have run this marathon of training, with steady determination and passion for a cause I love. And now, as I see the finish line in the distance, I feel as if I am about to finish the Great North Run alongside the sea, preparing to set sail on the next stage of my journey...

'To study the phenomena of disease without books is to sail an uncharted sea; while to study books without patients is not to go to sea at all.' -Sir William Osler

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ABBREVIATIONS

aCGH = array comparative genome hybridisation

AD = autosomal dominant

ALS = amyotrophic lateral sclerosis

AR = autosomal recessive

Asn = asparagine

BM = Bethlem myopathy

Ca^{2+} = calcium

CCC= concordance correlation coefficient

CDEs = Core Data Elements

cDNA = complementary deoxyribonucleic acid

CI = confidence interval

CMD = congenital muscular dystrophy

CNV = copy number variation

CO_2 = carbon dioxide

COX = cytochrome oxidase

CsA = cyclosporine A

CT = computed tomography

CyP = cyclophilin

DAPI = 4',6-diamidino-2-phenylindole

dbGaP = database of Genotypes and Phenotypes

DNA = deoxyribonucleic acid

dSMA = distal spinal muscular atrophy

EDS = Ehlers-Danlos syndrome

EK = Egen Klassifikation

EK2 = Egen Klassifikation, version 2

EM = electron microscopy

ER = endoplasmic reticulum

FBS = fetal bovine serum

FVC = forced vital capacity

GC = guanine-cytosine

gDNA = genomic deoxyribonucleic acid

Gly = glycine

GOSH = Great Ormond Street Hospital

H&E = haematoxylin and eosin

IF = immunofluorescence

IgG = immunoglobulin G

IHC = immunohistochemical

kb = kilobase

kDa = kilodalton

Leu = leucine

LOVD = Leiden Open Variation Database

Lys = lysine

Mg^{2+} = magnesium

Mb = megabase

MDC1A = merosin-deficient congenital muscular dystrophy

MIM = Mendelian Inheritance in Man

MRC = Medical Research Council

MRI = magnetic resonance imaging

NADH-TR = nicotinamide adenine dinucleotide-tetrazolium reductase

NGS = next generation sequencing

NHNN = National Hospital for Neurology and Neurosurgery

NIH = National Institutes of Health

nm = nanometre

NSCT = National Specialised Commissioning Team

PBS = phosphate buffered saline

PCR = polymerase chain reaction

PTC = premature termination codon

PTP = permeability transition pore

RF = rectus femoris

RT = reverse transcriptase

Ser = serine

SNP = single nucleotide polymorphism

TH = triple helical

UCL = University College London

UCMD = Ullrich congenital muscular dystrophy

UK = United Kingdom

USA = United States of America

VC = vital capacity

VL = vastus lateralis

VWA = von Willebrand factor type A

AIMS OF THIS THESIS

1. To report an improved understanding of the phenotypic stratification of the collagen VI-related myopathies resulting from natural history data collected as part of a large, retrospective international study.

2. To propose a revised diagnostic algorithm for assessing patients with phenotypes suggestive of collagen VI-related myopathies.

3. To evaluate the role of chromosomal microarrays and next generation/high-throughput sequencing, including whole-genome sequencing and exome sequencing, in evaluating patients with phenotypes suggestive of collagen VI-related myopathies.

4. To evaluate how histological and immunohistochemical findings in muscle biopsies of collagen VI-related myopathy patients serve as diagnostic tools and as well as potential indicators of the pathophysiology underlying the collagen VI-related myopathies.

This thesis is divided into 6 parts. Chapter 1 serves as an Introduction, providing background about the collagen VI-related myopathies. Chapters 2-5 discuss the aims listed above, and Chapter 6 serves as a Conclusion. Discussions are within Chapters 2-5 with a broader discussion included in Chapter 6.

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

The congenital muscular dystrophies (CMDs) are a clinically and genetically heterogeneous group of disorders characterised by the congenital onset of weakness and hypotonia, typically associated with dystrophic-appearing muscle biopsy findings (fibrosis with or without necrosis). In his description of the congenital muscular dystrophies in 1903, Dr Frederick Eustace Batten, a neurologist and paediatrician at Great Ormond Street Hospital noted, 'The disease is congenital or starts in early infancy, and is characterized by smallness, lack of power, and loss of tone in all the muscles of the body without localized atrophy or hypotrophy of individual muscles or groups of muscles. …The child usually learns to talk at the normal age, and intellectually is often in advance of his years.'[1] Over 105 years later, this description still holds true for many subgroups of CMD, and, in particular, is an appropriate clinical description of a CMD subgroup known as collagen VI-related myopathies which result from a decrease, absence or dysfunction of the extracellular matrix protein collagen VI.

Studies of the incidence of the CMDs as a group have been estimated as 4.7 per 100,000 (live births) in Northern Italy[2] and 6.3 per 100,000 live births in Western Sweden.[3] The increased awareness and recognition of the clinical phenotypes of the collagen VI-related myopathies has more recently resulted in the conclusion that this group of conditions is one of the most common forms of CMD.[4-6] A Northern England study of genetic muscle disease reported an estimated incidence of UCMD of 0.13 per 100,000 and BM of 0.77 per 100,000.[7] An Australian study of muscle biopsy immunohistochemistry studies performed in 101 patients with congenital muscular dystrophy estimated that collagen VI-related myopathies are the second most frequent CMD subtype after dystroglycanopathies in their cohort.[8]

Our neuromuscular centre (the Dubowitz Neuromuscular Centre), as the UK National Specialised Commissioning Team (NSCT) referral centre for congenital muscular dystrophies, offers a great opportunity to study a large CMD population. A recent retrospective review of the diagnostic outcomes of 214 patients referred to our neuromuscular centre for an evaluation of possible CMD between 2001-2008 revealed that collagen VI-related myopathies were the most common form of genetically-confirmed CMD, with a relative frequency of 19%, compared to

dystroglycanopathies and merosin-deficient CMD (MDC1A), with relative frequencies of 12% and 10% respectively.[9]

1.2 STRUCTURE AND ASSEMBLY OF COLLAGEN VI

Mutations in any of the three collagen VI genes (*COL6A1*, *COL6A2* or *COL6A3*) can affect the complex assembly and secretion of collagen VI, resulting in the spectrum of phenotypes seen in the collagen VI-related myopathies. *COL6A1* and *COL6A2* are located on chromosome 21q22.3,[10-11] and *COL6A3* is located on chromosome 2q37.[12] Collagen VI is a heterotrimeric monomer composed of three alpha chains: α1(VI), α2(VI) and α3(VI) encoded by *COL6A1*, *COL6A2* and *COL6A3* respectively[13-14] and each containing a short triple helical domain flanked by globular domains. The α1(VI) and α2(VI) chains are similar in size and structure, as both chains contain a 335 or 336 amino acid triple helix containing a glycine triplet repeat motif and contain one A type domain found in von Willebrand factor type A (VWA) domain N-terminal to the triple helix (N1) and two VWA domains C-terminal to the triple helix (C1 and C2). The α3(VI) chain is larger with 10 N-terminal domains (N1-N10) and two C-terminal VWA domains (C1 and C2). The α3(VI) chain also contains other C-terminal domains (C3-C5).[15] Assembly of collagen VI proceeds intracellularly with monomers aligning in an antiparallel fashion to form dimers (Figure 1). This initial chain association is thought to be mediated by C1 domains.[16-17] The dimers in turn align laterally to form tetramers. Disulfide bonds between cysteine residues found in the triple helix of all 3 collagen VI chains help to stabilize the dimers and tetramers.[18-20] The tetramers are then secreted extracellularly and align in an end-to-end fashion, forming beaded microfilaments as the final product of collagen VI assembly, which have a diameter of 4.5 nm and a periodicity of 100-105 nm.[18, 20-25]

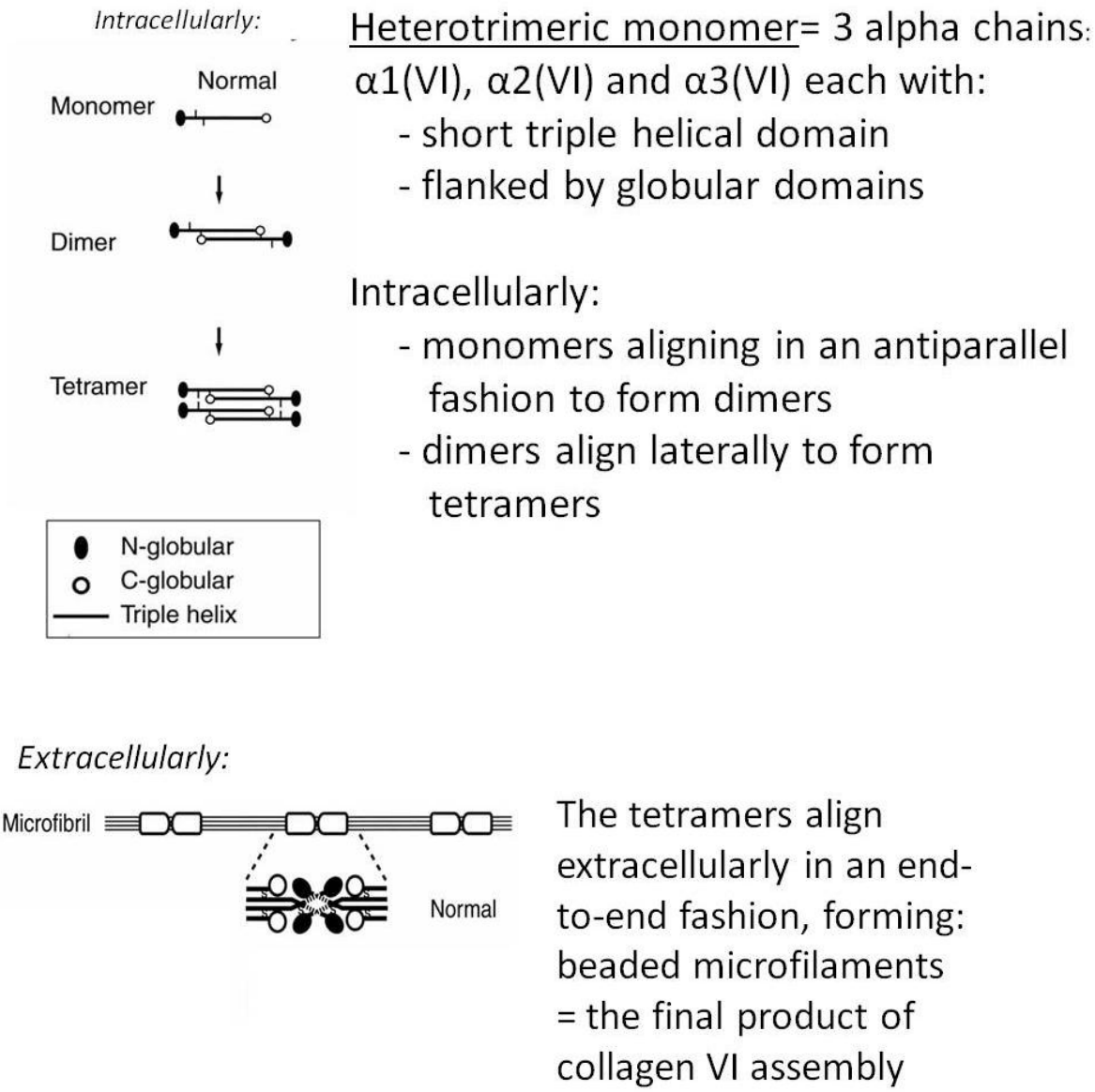


Figure 1: Graphic representation of the assembly of Collagen VI microfilaments.

(Modified from Pan TC et al. 2003[26] and reproduced with permission from Elsevier.)

The function of collagen VI as an extracellular matrix protein is not precisely understood. Some studies suggest that collagen VI helps to anchor the basement membrane to the pericellular matrix in muscle[27-29] while other studies suggest that collagen VI plays a role in cell signalling and cell migration.[30-31] Collagen VI interacts with multiple extracellular matrix proteins including perlecan,[32] fibronectin, decorin, biglycan,[33-34] collagen II,[34] collagen IV,[35] collagen XIV,[36] fibulin 2,[37] hyaluran[38-39], heparin,[40] microfibril-associated glycoprotein 1[41] and membrane-associated chondroitin sulfate proteoglycan 4[42] (Figure 2). Certainly the full extent of the complex and numerous interactions collagen VI has with neighbouring extracellular matrix proteins and cell surface receptors have yet to been fully elucidated. (See Chapter 5, section 5.2 for a further discussion of collagen VI and its muscle extracellular matrix neighbours.)

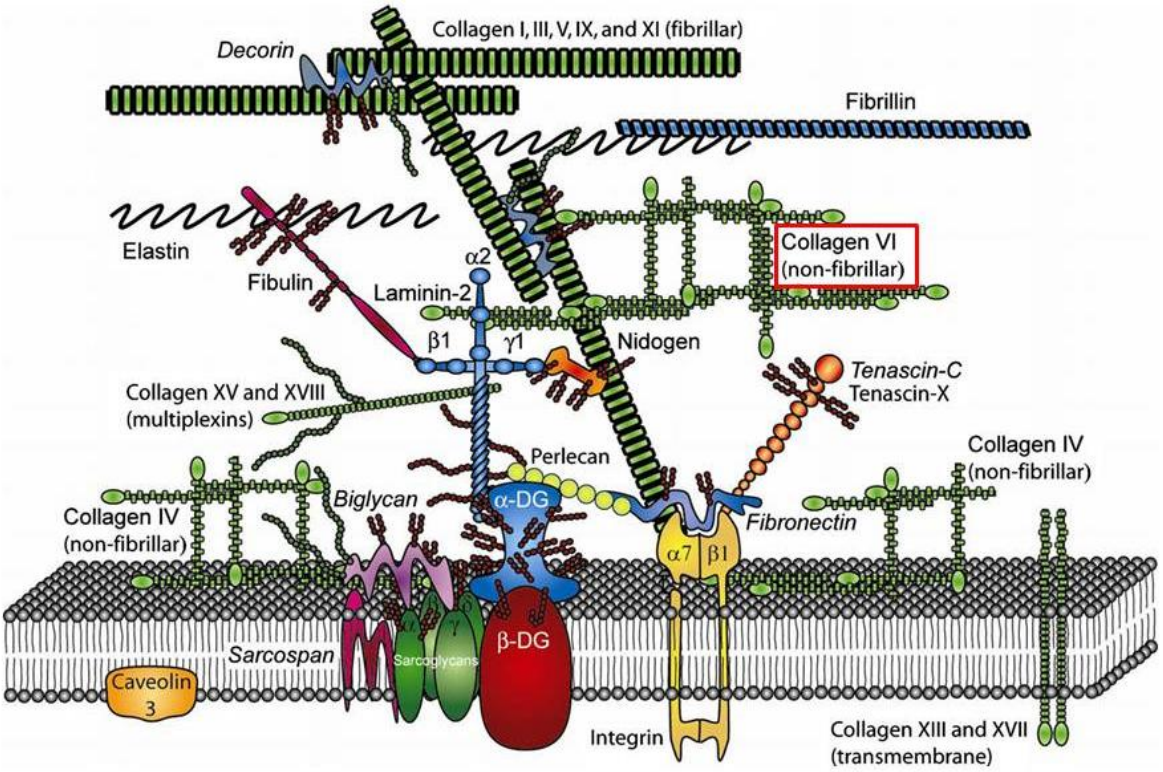


Figure 2: Schematic of the muscle extracellular matrix (ECM).

(Figure adapted from Voermans et al. 2008[11] and reproduced with permission from Elsevier.)

1.3 COLLAGEN VI-RELATED MYOPATHY PHENOTYPES

1.3.1 Ullrich congenital muscular dystrophy

Ullrich congenital muscular dystrophy (UCMD [MIM 254090]), Bethlem myopathy (BM [MIM 158810]) and intermediate collagen VI-related myopathy form a subgroup within the congenital muscular dystrophies known as the collagen VI-related myopathies.[43] Ullrich congenital muscular dystrophy was first described in 1930 by Dr Otto Ullrich, who termed the condition 'Skleratonische Muskeldystrophie' (scleratonic muscular dystrophy), noting evidence of congenital weakness associated with proximal joint contractures and joint laxity.[44-45] UCMD results from recessively or dominantly acting mutations in any of the three collagen VI genes (*COL6A1*, *COL6A2* or *COL6A3*).[26, 46]

The first signs of Ullrich congenital muscular dystrophy can manifest in utero with decreased fetal movement frequently reported.[43-45, 47-48] At birth, UCMD patients classically demonstrate hypotonia, proximal joint contractures, hip dislocation(s), prominent calcanei and distal hyperlaxity resulting in abnormal positioning of the hands and feet (with hands in a position of wrist flexion and resting against the ventral surface of the forearms and feet in a position of dorsiflexion and resting against the anterior surface of the shins). Torticollis and kyphoscoliosis can often be seen at birth, as well.[43, 48-50]

While children with UCMD may achieve independent ambulation, this ability is lost in early childhood.[43, 48-50] Some patients attain the ability to walk on their knees only, often related to progressive knee contractures. After becoming wheelchair-dependent, most UCMD patients demonstrate relatively stable muscle weakness while joint contractures continue to progress, compounding the muscle weakness and significantly contributing to their overall level of disability.[51]

Early-onset of an invariable decline in respiratory function necessitating the initiation of night-time non-invasive ventilation is a salient clinical feature of UCMD,[43, 48, 52] and is discussed in detail in Chapter 2. It is essential to note that in the past, the failure to recognise respiratory insufficiency and implement non-invasive ventilation has resulted in the untimely death of UCMD patients during the teenage years.[48] For this reason, knowledge of the natural history of UCMD past the late teenage years in

patients in whom respiratory support (non-invasive ventilation) has been appropriately initiated, had been limited.[51]

While primary cardiac involvement has not been associated with UCMD, autopsy evidence of *cor pulmonale* (right heart failure) has been reported in an Italian UCMD patient secondary to untreated respiratory failure.[53] Right ventricular hypertrophy was also noted in a patient in the States in whom the non-invasive ventilation parameters were not appropriately adjusted to meet the patient's respiratory function needs over time (unpublished report; Dr Carsten Bönnemann).

Transient feeding difficulties necessitating nasogastric tube feedings can be evident in the newborn period[52] or present later with failure-to-thrive necessitating gastrostomy tube feeds.[49] While some UCMD patients demonstrate spinal stiffness without an evident spinal curvature, the vast majority develop progressive scoliosis, requiring surgical intervention/spinal instrumentation during the first decade of life. The onset of scoliosis typically precedes the loss of ambulation in UCMD, and can appear as early as the preschool years or even congenitally.[52, 54]

Preserved intelligence is another noteworthy feature of Ullrich congenital muscular dystrophy. In particular, UCMD patients tend to excel academically and continue education onto the graduate level (personal experience in the US and the UK).

1.3.2 Bethlem myopathy

Bethlem myopathy (BM) is characterized by slowly progressive muscle weakness and distal joint contractures and was first described in 1976 by Drs Jaap Bethlem and George K. van Wijngaarden.[55] The identification of causative mutations in *COL6A1*, *COL6A2* and *COL6A3* in Bethlem myopathy patients resulted in BM being recognized as the first collagen VI-related myopathy.[56-59] Whilst BM typically follows autosomal dominant inheritance, rare autosomal recessive inheritance has been described as well.[60-61] One particular recessive mutation, a homozygous nonsense mutation in *COL6A2* described in a consanguineous family, has been associated with a specific Bethlem myopathy phenotype termed 'myosclerosis,' which manifests with significant contractures, only mild muscle weakness and what is described as a 'woody' feel to the muscles.[62]

While Bethlem myopathy is often described as a slowly progressive myopathy of adulthood, its categorisation within the congenital muscular dystrophies likely relates, in part, to the fact that symptoms of Bethlem myopathy can present as early as birth. Symptom onset during infancy in BM was reported in a study of 23 children (from 7 different families) diagnosed with Bethlem myopathy (based on autosomal dominant inheritance, generalised onset of weakness and joint contractures either in infancy or early childhood and a myopathic-appearing muscle biopsy). In particular, early symptoms reported include hypotonia, neck flexion weakness and contractures. The BM patients included in this study typically demonstrated a Gowers' manoeuvre when arising from the floor and a delayed onset of walking. In particular, 18/23 (78%) of the BM patients evaluated as part of this study demonstrated neuromuscular symptoms within the first 2 years of life. Evidence of hypermobility of the wrists and fingers in children with BM, subsequently evolving into flexion contractures, is also highlighted in this study.[63]

Progressive contractures of the Achilles tendons and elbows usually manifest by the end of the first decade in patients with Bethlem myopathy. While long finger flexor tightness can be evident in BM patients during childhood (personal experience), their progression becomes most apparent during adulthood, typically resulting in patients being unable to fully extend their fingers when the wrist is 'dorsiflexed,' a well-recognised sign of Bethlem myopathy[49] which has been referred to as the 'Bethlem sign.'[51] Some patients with BM may develop scoliosis. Progressive stiffening of the spine can be seen, as well.

BM patients develop proximal muscle weakness but typically maintain the ability to ambulate into adulthood. By 50 years of age, however, more than 2/3 of BM patients rely on the use of a wheelchair to aide ambulation, classically for outdoor use while independent ambulation indoors is usually maintained.[63] In most BM patients it is the progression of joint contractures, in combination with predominantly limb-girdle weakness, which may ultimately limit independent ambulation. Some BM patients undergo surgical release of the Achilles tendons which can, at least for a period of time, stabilise walking ability by improving ankle mobility.

Respiratory insufficiency necessitating night-time ventilation can occur in late adulthood in Bethlem myopathy patients but can be quite variable.[59, 64] As detailed in Chapter 2, the results of our recent international study of pulmonary function in the collagen VI-related myopathies indicate that the need for non-invasive ventilation in

adult Bethlem myopathy patients is rare, with only 1/43 (2%) of the BM patients studied having initiated night-time non-invasive ventilation.

1.3.3 Intermediate collagen VI-related myopathy

Ullrich congenital muscular dystrophy and Bethlem myopathy were initially viewed as two separate phenotypic entities. Subsequently, however, it has become apparent that there is indeed a phenotypic spectrum of conditions resulting from a deficiency or aberrant formation of collagen VI. While UCMD patients fall along the severe end of this spectrum and BM patients fall at the mild end of this spectrum, in between fall patients with phenotypes intermediate to UCMD and BM, referred to as having 'intermediate' collagen VI-related myopathy.[51, 53] The phenotypic data gathered as part of a large-scale international study of collagen VI-related myopathy patients (Chapter 2) has enabled me to define the parameters of the intermediate collagen VI-related myopathy phenotypic category, based on both motor function and pulmonary function. In particular, intermediate collagen VI-related myopathy patients maintain ambulation until the late teenage or early adult years and have a profile of decline in pulmonary function similar to UCMD patients, albeit with an onset slightly after that seen in UCMD patients.

Prior to efforts to define this 'intermediate' collagen VI-related myopathy category, patients with this phenotype were diagnosed with either 'mild UCMD' or 'severe BM.'[53] For this reason, determining an accurate frequency for patients within the intermediate collagen VI-related myopathy category is challenging. The data gathered as part of a large international natural history study of collagen VI-related myopathy patients (Chapter 2) was carefully analysed and patients categorised as UCMD, BM or intermediate collagen VI-related myopathy according to their profiles of motor function and pulmonary function. Of the 145 molecularly confirmed collagen VI-related myopathies patients with pulmonary function data available, 75 patients (52%) had a phenotype consistent with UCMD, 43 patients (30%) had a phenotype consistent with BM and 27 patients (19%) had a phenotype consistent with intermediate collagen VI-related myopathy. Based on this data, one could hypothesise that the frequency of collagen VI-related patients falling within the intermediate category may be significantly less than the frequency of UCMD patients and BM patients (Figure 3).

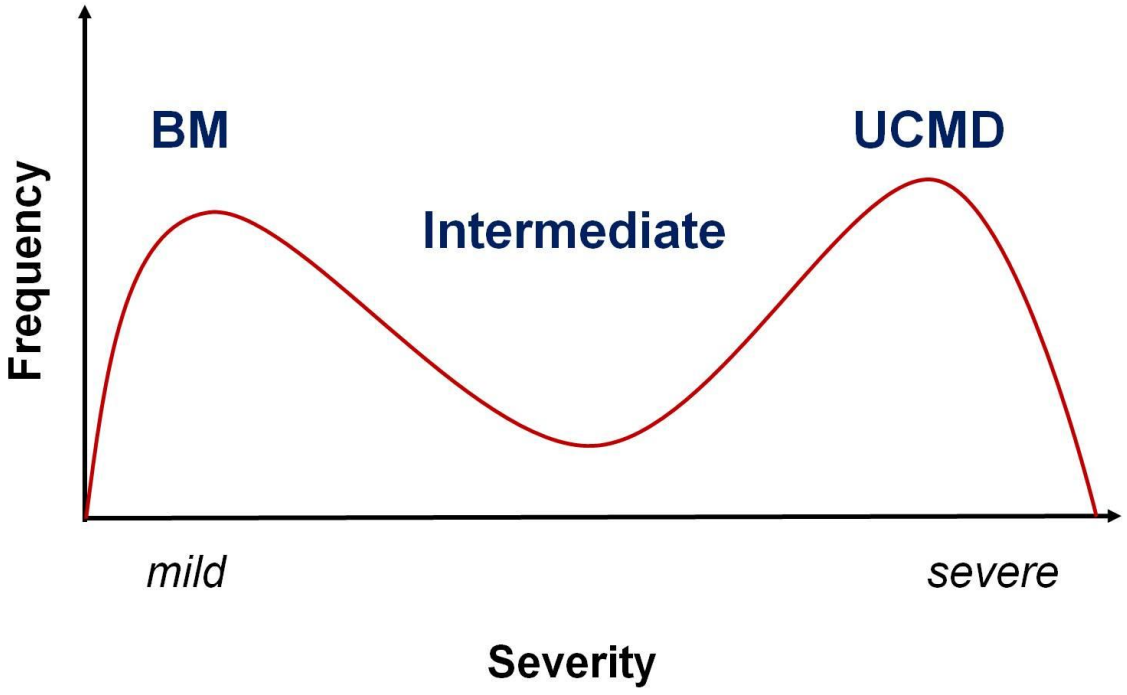


Figure 3: Graphical representation of a working hypothesis on the phenotypic spectrum of the collagen VI-related myopathies.

1.3.4 Skin findings in the collagen VI-related myopathies

As collagen VI is also expressed in the skin, collagen VI deficiency results in skin findings. These findings can be striking and, as such, can aid in arriving at a clinical diagnosis of collagen VI-related myopathy. Unlike motor function and pulmonary function, which manifest differently in UCMD, BM and intermediate collagen VI-related myopathy patients, the skin findings seen in the collagen VI-related myopathies do not appear to be phenotype-specific, per se.

In fact, most collagen VI-related myopathy patients have some abnormal skin findings. These findings include keratosis pilaris (or follicular keratosis) typically along the extensor surfaces of the arms and legs, abnormal scar formation (including keloid scars and atrophic or 'cigarette paper' type scars) as well as striae formation.[65-66] 'Spontaneous' keloids, that is, keloids occurring along sites on the skin which have not been previously injured, have been observed in patients with Bethlem myopathy.[67] Other skin findings include soft skin on the palms of the hands and feet as well as a fine 'criss-cross' pattern of creases along the palms of the hands and feet, which can also be seen in patients with Ehlers Danlos syndrome.[51]

1.4 GENOTYPE-PHENOTYPE CORRELATIONS

One might hypothesise that analogous to the spectrum of clinical phenotypes in the collagen VI-related myopathies there may be a molecular spectrum of mutations resulting in varying degrees of collagen VI expression and correlating with particular phenotypes. Despite multiple attempts at establishing genotype-phenotype correlations, the identification of different collagen VI-related myopathy phenotypes resulting from identical *COL6A1, COL6A2* and *COL6A3* mutations have complicated these efforts and have highlighted the molecular complexity underlying these conditions. Among the large number of mutations identified in the collagen VI genes, however, a number of mutational mechanisms as well as locations of mutations in specific regions of the *COL6A1, COL6A2* and *COL6A3* genes have been recognised as correlating with severity of phenotype.

As stated above (section 1.3), both autosomal recessive (AR) and autosomal dominant (AD) mutations in *COL6A1, COL6A2* and *COL6A3* have been found to

underlie the spectrum of collagen VI-related myopathies. For this reason, pursuing parental *COL6A1, COL6A2* and *COL6A3* sequencing is essential in determining the mode of inheritance in collagen VI-related myopathy probands.

In BM, the most common mode of inheritance is autosomal dominant; however, I and others have reported rare autosomal recessively inherited Bethlem myopathy.[60-61] Sequencing performed in these families demonstrated that *COL6A2* frameshifting mutations resulting in premature termination codons (truncating mutations) do not confer a phenotype of collagen VI-related myopathy when occurring in heterozygosity, as in the case of asymptomatic parents of children with Bethlem myopathy. The occurrence of a *COL6A2* truncating mutation in compound heterozygosity with a *COL6A2* missense mutation, however, results in a phenotype of Bethlem myopathy.[60-61] Without efforts devoted to carefully deciphering modes of inheritance in collagen VI-related myopathies, accurate genetic counselling may not be possible.

1.4.1 Common mutational mechanisms in collagen VI-related myopathies

The most common mutational mechanism resulting in a phenotype of UCMD (in non-consanguineous populations) is *de novo* autosomal dominant.[6, 26, 68-69] In order to form the collagen VI heterotrimeric or triple helical monomer, the α1(VI), α2(VI) and α3(VI) collagen chains are believed to fold from their respective C terminal ends. Further assembly into dimers and tetramers is reliant on two essential cysteine residues in the N terminal region of the triple helical (TH) domain of each monomer. A strong 'dominant negative' effect from *de novo* autosomal dominant mutations can result when in-frame skipping of exons in the N-terminal region of the collagen VI α chains does not disrupt the essential cysteine residues for dimer and tetramer formation. As a result, mutant and normal monomers assemble into dimers and tetramers, so that only 1/4 of the dimers and 1/16 of the tetramers are composed entirely of normal chains. Since 15/16 tetramers contain mutant chains, and mutant tetramers cannot properly align with normal tetramers, the final assembly of collagen VI microfibrils cannot proceed. Collagen VI mutations causing skipping of exon 16 of *COL6A3* are the most common of the *de novo* autosomal dominant mutations and result in a severe UCMD phenotype.[69-70]

A milder 'dominant negative' effect can result from *de novo* autosomal dominant mutations which disrupt the essential cysteine residues, preventing the formation of monomers and dimers with abnormal collagen VI chains. As a result, all tetramers secreted are composed of normal collagen VI chains, albeit at less than 1/2 the quantity of controls.[26] Collagen VI mutations resulting in skipping of exon 14 in *COL6A1* are an example of this milder type of 'dominant negative' mutation and result in collagen VI-related myopathy phenotypes milder than UCMD. In particular, exon skipping mutations of exon 14 in *COL6A1* are the most common mutation found in Bethlem myopathy patients.[26, 71-75]

Another form of 'dominant negative' mutation which commonly occurs in the collagen VI-related myopathies are so-called 'glycine mutations.' These mutations result in substitutions of single amino acids for glycine in the Gly-X-Y motifs of the highly conserved N-terminal triple helical domain of any of the collagen VI chains[57, 73-74, 76-77] and allow assembly of the affected collagen VI chain into triple helical monomers. The result of glycine mutations, both molecularly and clinically, depend on the location of the amino acid substitution.[78] As such, glycine mutations have been identified in autosomal dominantly inherited Bethlem myopathy as well as *de novo* autosomal dominant intermediate collagen VI-related myopathy and UCMD.[6, 54, 78]

Autosomal recessive inheritance occurs in UCMD, typically by mutational mechanisms such as nonsense mutations, intragenic deletions and splice site mutations (resulting in out-of-frame transcripts) and mutations near the C terminal end of the triple helical domain[69] which result in a 'functional null' alleles.[27, 46, 79] Although not as common, homozygous missense mutations can result in UCMD.[80] A recent clinical, cellular and molecular study of 49 collagen VI-related myopathy patients reported that homozygous mutations either before or within the triple helical domains which result in premature termination codons (PTCs) were associated with the most severe UCMD phenotypes (patients who never achieved independent ambulation).[81]

While intermediate collagen VI-related myopathy patients have been found to harbour either autosomal dominant or autosomal recessive mutations in *COL6A1, COL6A2* or *COL6A3*, exon skipping mutations or *de novo* autosomal dominant mutations involving glycine residues are two common mutational mechanisms which result in this collagen VI-related myopathy phenotype.

1.4.2 Biochemical consequences of collagen VI mutations

The sequencing of *COL6A1*, *COL6A2* and *COL6A3*, a combined total of 107 coding exons, in patients being evaluated for collagen VI-related myopathies, has revealed a large number of polymorphic variants of unknown significance.[74] Given the uncertain significance of these variants, further genetic as well as biochemical analyses are essential for determining potential pathogenicity. One means of studying the biochemical consequences of dominant or recessive collagen VI mutations is by analysing collagen VI biosynthesis. One such study discovered that the α2(VI) C1 domain is not essential for dimer formation,[82] while other studies found that this domain is critical for microfibril formation.[80, 83]

In vitro biochemical studies of collagen VI have revealed alternative splicing of *COL6A2*, which results in 3 collagen VI α2 chains: α2C2, α2C2a and α2C2a' with α2C2 recognised as the major species.[84] A study of the role of the C2a splice variant demonstrated that in an UCMD patient with recessively inherited homozygous mutations in the C2 domain of *COL6A2*, fibroblasts secreted collagen VI protein exclusively composed of the α2C2a splice variant. The authors hypothesise that the use of the α2C2a splice variant may be a compensatory measure due to the absence of normally formed collagen VI α2 chain. Since the reported patient's clinical severity is less than would be expected from the mutational and biochemical mechanisms, and may be related to the increased use of the α2C2a splice variant, this study highlights how clinical severity cannot be predicted based on biosynthetic abnormalities.[80]

1.4.3 Recently-described human collagen VI genes (*COL6A4, COL6A5* and *COL6A6)*

In 2008 three new collagen VI genes were identified on chromosome 3q22.1: *COL6A4, COL6A5* and *COL6A6*, corresponding to murine collagen VI α4, α5 and α6 chains. The human *COL6A4* gene is disrupted by a chromosome break, which results in functional inactivity. *COL6A5* mRNA expression is restricted to a few tissues and is not expressed in muscle. *COL6A6* is reportedly expressed in many tissues including heart and skeletal muscle.[15] Given that the collagen VI α4, α5 and α6 chains demonstrate high homology to the collagen VI α3 chain and collagen VI

knockout mice (*Col6a1*$^{-/-}$) do not express the collagen VI α4, α5 or α6 chains, it has been hypothesised that perhaps the collagen VI α5 or α6 chains substitute for the collagen VI α3 chain.[85] It is important to note, however, that to date no patient with collagen VI deficiency without mutations in *COL6A1, COL6A2* or *COL6A3* has been identified to have a mutation in *COL6A6.*

1.5 POTENTIAL PATHOPHYSIOLOGICAL MECHANISMS / THERAPEUTIC TARGETS

Initial insights into potential pathophysiologic mechanisms underlying the collagen VI-related myopathies originated from *in vitro* and *in vivo* studies performed on a mouse model of collagen VI deficiency (*Col6a1*$^{-/-}$)[30, 86-88] as well as in muscle biopsy specimens and myoblast cultures from collagen VI-related myopathy patients.[89-90] Evidence of mitochondrial dysfunction, which was discovered along with increased apoptosis in patient-derived myoblast cultures, first implicated mitochondrial dysfunction as a pathophysiologic mechanism in the collagen VI-related myopathies. Further studies sought to understand how the deficiency of an extracellular matrix protein might result in mitochondrial dysfunction. Studies in the *Col6a1*$^{-/-}$ mouse revealed loss of muscle contractile force with associated ultrastructural abnormalities of the sarcoplasmic reticulum (SR) and mitochondria and apoptotic changes. A reversal of these ultrastructural changes and a decrease in apoptotic nuclei occurred when *Col6a1*$^{-/-}$-derived myofibres were plated on purified collagen VI or by treatment with cyclosporin A (CsA).[30] Given that CsA inhibits the mitochondrial permeability transition pore (PTP), these findings supported mitochondrial dysfunction as a pathophysiology underlying collagen VI deficiency. Studies performed in patient myoblasts further supported mitochondrial dysfunction, with evidence that the mitochondrial PTP threshold was low, predisposing the mitochondrial PTP to aberrant opening and consequently ATP depletion.[90]

A subsequent study of genetic ablation of cyclophilin (CyP) D in *Col6a1*$^{-/-}$mice demonstrated a normalisation of apoptotic rates and ultrastructural appearances of SR and mitochondria, thus providing evidence suggesting that it is cyclosporine A's inhibition of CyP D which rescues the muscle alterations in *Col6a1*$^{-/-}$ mice.[91] Besides inhibiting cyclophilin D, cyclosporin A inhibits calcineurin, as well, resulting in immunosupression. D-MeAla^3-EtVal^4-cyclosporin (Debio 025) is a selective

cyclophilin D inhibitor which does not have the calcineurin inhibiting properties of CsA. A study of treatment of *Col6a1*$^{-/-}$mice with D-MeAla^{3}-EtVal^{4}-cyclosporin (Debio 025) demonstrated desensitisation of the mitochondrial permeability transition pore and prevention of muscle cell apoptosis, providing evidence that this compound has promising therapeutic implications for patients with collagen VI-related myopathies.[92]

More recently, studies performed in the *Col6a1*$^{-/-}$ mouse have revealed that increased apoptosis as well as ultrastructural alterations of sarcoplasmic reticulum and mitochondria result from defective autophagy (the process by which the cell forms cytoplasmic autophagosomes to deliver to lysosomes). Restoration of normal autophagic flux either by a low protein diet or treatment with rapamycin or cyclosporine A improved structural abnormalities of the SR and mitochondria, blocked apoptosis and induced autophagy with concomitant improvement of muscle strength in the *Col6a1*$^{-/-}$ mouse.[93]

While the *Col6a1*$^{-/-}$ mouse skeletal muscle demonstrates findings similar to human collagen VI-related myopathy patient muscle, including a complete absence of collagen VI expression, the phenotype of the *Col6a1*$^{-/-}$ mouse is that of a very mild myopathy involving skeletal muscles and the diaphragm. Given the inability of the *Col6a1*$^{-/-}$ mouse to accurately recapitulate the human clinical phenotype, the use of another animal model has been investigated. Using morpholinos to exon 9 of *col6a1*, a zebrafish model of UCMD was created, while morpholinos to exon 13 of *col6a1* were used to create a zebrafish model of a milder collagen VI-related myopathy, similar to BM.[94] (Exon 9 is equivalent to the human *COL6A1* exon 10, which when deleted results in a truncated collagen VI α1 transcript and causes a phenotype of UCMD by way of a dominant negative mechanism. Exon 13 is equivalent to exon 14 in the human *COL6A1* gene, with skipping of this exon being the most common mutational mechanism resulting in Bethlem myopathy.) Both the zebrafish generated with exon 9 morpholinos and those with exon 13 morpholinos demonstrated dystrophic-appearing muscle with myofibre damage and evidence of apoptosis. Ultrastructurally, the mitochondria were swollen in appearance and the endoplasmic reticulum appeared dilated. There was also evidence of thinning of the sarcolemmal membrane along with membrane discontinuities. (Overall, all findings were more severe in the UCMD zebrafish model, created with the exon 9 morpholino than in the milder, BM-like zebrafish model created with the exon 13 morpholino.)[94]

Treatment of these two zebrafish models with CsA decreased apotosis and abnormal mitochondria but did not improve the myofibre integrity or repair the sarcolemmal membrane damage evident in the morphant zebrafish model. The improvement of motor function in both the zebrafish created with morpholinos designed to exon 9 of *col6a1* as well as those designed to exon 13 of *col6a1* further supports a role for cyclosporin A- or other cyclophilin D inhibitors- in the treatment of collagen VI-related myopathies. This study highlights, however, the clear need for another treatment strategy aimed at improving myofibre integrity and repairing sarcolemmal damage evident in these zebrafish models of collagen VI-related myopathy.[94]

1.6 DISCUSSION

The collagen VI-related myopathies have been recognised as a common form of congenital muscular dystrophy (CMD), and indeed have the highest relative frequency among genetically-confirmed CMDs in the UK.[9] Despite the often striking mixture of muscle and connective tissue features which manifest in patients with a deficiency of the myomatrix protein collagen VI, the diagnostic journey for patients with collagen VI-related myopathies can be challenging, particularly for those patients with mild and moderate phenotypes.

A primary goal of this clinical research is to better define the phenotypic spectrum of the collagen VI-related myopathies with a particular focus on delineating the category of patients falling between Ullrich congenital muscular dystrophy and Bethlem myopathy. In particular, given that unrecognised respiratory failure in patients with moderate-to-severe collagen VI-related myopathy carries a risk of high morbidity, as well as a risk of untimely death in the teenage to young adult years, studying and reporting the natural history of respiratory insufficiency in this patient population is imperative. Furthermore, robust natural history data is essential for optimising patient care as well as preparing for upcoming experimental clinical trials.

The journey toward clinical trials is dependent on an understanding of the molecular mechanisms underlying the clinical manifestations of disease. An understanding of the mechanisms by which mutations in *COL6A1, COL6A2* and *COL6A3* affect the complex assembly of the collagen VI microfibrils and manifest in a spectrum of collagen VI-related myopathy phenotypes has informed the generation of mouse and

zebrafish models. Despite studies performed on various collagen VI-related myopathy animal models, as well as parallel studies using patient-derived myofibres and myoblast cultures, the exact mechanism by which a deficiency of the extracellular matrix protein collagen VI results in abnormal mitochondrial function and abnormal autophagy remains unclear. Promising results from studies of Debio-025 in the collagen VI mouse model (*Col6a1*$^{-/-}$) offer hope for future experimental clinical trials in the collagen VI-related myopathies. Whether a reversal of apoptosis and an improvement in mitochondrial function alone are sufficient to affect a stabilisation of disease, or rather a therapeutic approach combining an anti-apoptotic compound with an agent which improves myofibre integrity and stabilises the sarcomere will be necessary to affect clinical improvements in collagen VI-related myopathy patients, remains to be seen.

First and foremost, the journey toward clinical trials begins with the careful phenotypic classification of patients, without which an accurate understanding of corresponding natural history would not be possible. Chapter 2 reports the results of a study of a large international series of collagen VI-related myopathy patients, which clarifies the phenotypic classification of the collagen VI-related myopathies with a particular focus on a leading cause of morbidity and mortality in this patient population, respiratory insufficiency.

CHAPTER 2: NATURAL HISTORY OF THE COLLAGEN VI-RELATED MYOPATHIES

2.1 INTRODUCTION

There has been a need for larger scale natural history studies to better understand the spectrum of collagen VI-related myopathy phenotypes, particularly given the diversity of terms used to describe the collagen VI-related myopathy phenotypes among different international centres and the lack of clear guidelines for distinguishing between these overlapping phenotypes. Robust natural history studies are an essential step for clarifying and validating phenotypic classifications, optimising clinical care and preparing for clinical trials. The internationally recognised need of optimising and standardising care in the congenital muscular dystrophies (CMDs)[95] along with the development of potential therapies for the collagen VI-related myopathies[89, 92] has highlighted the need for identifying relevant and viable outcome measures for future clinical trials in this patient population. The recognition that the collagen VI-related myopathies are one of the most common forms of congenital muscular dystrophy [4-6, 96] emphasises the importance of better defining the natural histories of this group of conditions.

Significant, progressive joint contractures complicate assessments of motor function and muscle strength in collagen VI-related myopathy patients, particularly those patients with UCMD and intermediate phenotypes. At the same time, previous reports and smaller case series have highlighted the frequent occurrence of respiratory failure in this patient population as arguably the most important aspect of the natural history as it is relevant to disease progression, mortality, and morbidity. Forced vital capacity (FVC) is a quantitative measure of pulmonary function which can be reliably measured in patients over 6 years of age, regardless of the severity of joint contractures and, therefore, may provide a good tool to chart the disease course and to better define the clinical subtypes.

While a reduction in vital capacity is an index of respiratory insufficiency, the degree of reduction is believed to be predictive of the presence of sleep disordered breathing (a decreased capacity to compensate for sleep-related decrease in alveolar ventilation). In particular, the consensus statement of the 117th ENMC Workshop on Ventilatory Support in Congenital Neuromuscular Disorders - Congenital Myopathies, Congenital Muscular Dystrophies, Congenital Myotonic Dystrophy and SMA (II) concluded that a vital capacity (VC) below 60% predicts the onset of sleep disordered breathing, and a VC below 40% predicts the presence of sleep hypoventilation.[97] Respiratory muscle testing has not yet been studied in detail in the

congenital muscular dystrophy population. A correlation between volitional respiratory muscle testing, nonvolitional testing and forced vital capacity has been established in other neuromuscular disorders of childhood, however, indicating that FVC can function as a reasonable indicator of global respiratory function.[98]

A UK retrospective study of 13 patients with Ullrich congenital muscular dystrophy reported a pattern of early and invariable decline in pulmonary function beginning at 6 years of age.[52] A more comprehensive natural history study of respiratory insufficiency assessing a large cohort of collagen VI-related myopathy patients has never been performed, however. Furthermore, the pulmonary function of collagen VI-related myopathy patients falling in the mild-to-moderate end of the phenotypic spectrum have not been studied in detail. We sought to evaluate longitudinal pulmonary function data (in the form of FVC values) to determine if profiles of decline in pulmonary function could help in clarifying the natural histories of the various phenotypes within the collagen VI-related myopathies and in improving anticipatory clinical care. Here I report the findings from a large, international retrospective study of pulmonary function in the collagen VI-related myopathies which I coordinated, the first study of this type and size in an international cohort of congenital muscular dystrophy patients.

2.2 METHODS

2.2.1 Patients

I coordinated the collection of phenotypic data from collagen VI-related myopathy patients with molecularly and/or biochemically confirmed diagnoses by liaising with neuromuscular specialist working at 10 different neuromuscular centres worldwide: United States (2), United Kingdom (2), Australia (2), Italy (2), France (1), and Belgium (1). Patients were considered to have molecular confirmation when the diagnosis was confirmed genetically (with pathogenic mutation/s in *COL6A1, COL6A2* or *COL6A3*), while biochemical confirmation indicates a diagnosis based on evidence of significantly decreased or mislocalised collagen VI on muscle biopsy immunohistochemical studies. Retrospective chart reviews were performed by collaborating neuromuscular specialists (see Collaborators, page 5) in accordance

with ethical guidelines of each participating neuromuscular centre. Spirometry techniques were performed according to international standards.[99]

I had initiated this research with the collection of phenotypic data on a cohort of collagen VI related myopathies referred to the Children's Hospital of Philadelphia (from many areas of the US). Recognizing that such data would be significantly more powerful in defining the natural history of the collagen VI-related myopathies if expanded to other cohorts of collagen VI-related myopathy patients, I relocated to the Dubowitz Neuromuscular Centre at the Great Ormond Street Hospital, a National Specialised Commissioning Team (NSCT) for congenital muscular dystrophies. Here I began working on gathering comprehensive phenotypic data from a large cohort of collagen VI-related myopathy patients referred to our NSCT centre from across the UK. Consequently, I was able to personally collect data from the patients followed at 2 participating neuromuscular centres (Great Ormond Street Hospital for Children, London, UK and The Children's Hospital of Philadelphia, Philadelphia, USA) and coordinate the collection of data from collaborators at 8 further specialised neuromuscular centres internationally.

While it had become clear that phenotypes of collagen VI-related myopathy intermediate to UCMD and BM exist, this phenotypic category had not been clearly defined. To address the natural variation amongst neuromuscular specialists in clinically categorizing patients as intermediate collagen VI-related myopathy versus Bethlem myopathy, the patients designated as having either Bethlem myopathy or intermediate collagen VI-related myopathy were initially studied as one group. Patient forced vital capacity measurements were plotted in order to evaluate individual patient patterns of FVC over time. In studying individual trends of FVC measurements over time in this group, two clear subgroups were apparent: one group whose FVC values demonstrated continued decline beginning at approximately 7 years of age and another group whose FVC values either remained stable or improved over time. When the maximal motor function data of the patients in these two subgroups was studied, it was found that none of the patients with progressive decline in pulmonary function (measured by FVC) ever achieved the ability to run or hop; these patients were then assigned to the phenotypic category of intermediate collagen VI-related myopathy. The group of patients with either stable or improving FVC values typically achieved the ability to run or hop and were assigned the phenotypic category of Bethlem myopathy.

Due to the fact that forced vital capacity data was collected retrospectively from ten different neuromuscular centres spanning 6 countries and 3 continents, spirometry machines varied and; therefore, percent predicted FVC values were derived from the reference equations specific to the spirometry machines used. I attempted to gather 'raw' FVC data (FVC in litres) corresponding to each percent predicted FVC value provided, with the goal of utilising the same formula for converting the 'raw' FVC data into percent predicted values. I discovered, however, that 'raw' FVC data (in litres) was not available for a large number of FVC measurements. Rather than discard a large number of FVC values, I (in consultation with a statistician at the National Institutes of Health, Bethesda, Maryland, USA) decided to analyse the percent predicted FVC values provided by each centre as derived from their respective spirometry machines.

2.2.2 Statistical analyses

Statistical analyses were performed in consultation with a statistician (S. Auh; see Collaborators, page 5) from the National Institute of Neurological Disorders and Stroke (NINDS)/National Institute of Health (NIH), USA). Linear mixed models were used to examine the effect of an independent variable (or variables) of interest on forced vital capacity (FVC). The analysis was based on multiple FVC values per subject. A working covariance structure was assumed as intraclass correlation covariance structure in order to take into account correlations among different number of FVC values per subject. Analyses were implemented in SAS (SAS institute Inc., Cary, NC) using PROC Mixed [100] to conduct linear mixed models and PROC Lifetest to generate graphs for time to events data. A concordance correlation coefficient (CCC), proposed by Vonesh et al[101] for a goodness-of-fit measure in linear mixed model was calculated in order to check the adequacy of the linear mixed models. Summary statistics for FVC were described by using mean $\pm$ standard deviation. All statistical tests were conducted with a significance level of 0.05.

2.3 RESULTS

From a cohort totalling 211 molecularly confirmed collagen VI-related myopathy patients originating from 10 international neuromuscular centres, 486 forced vital

capacity measurements were recorded in 145 patients. The total number of FVC measurements collected per patient ranged from 1 to 14 measurements (mean= 3.35; median= 3). Of the 145 patients with FVC data 80 (55%) were male and 65 (45%) were female. Seventy-five patients (52%) were diagnosed with Ullrich CMD (Table 1). FVC data from 13 patients followed at the Dubowitz Neuromuscular Centre and 13 patients followed at the Neuromuscular Centre, Garches (France) had been described previously.[52, 54]

The relationship between forced vital capacity and phenotype was highly significant ($p < 0.0001$) with the distribution of FVC values demonstrating a direct relationship with severity of clinical phenotype (Figure 4). The relationship between age and FVC for UCMD and intermediate patients was also highly significant ($p < 0.0001$) with UCMD patients demonstrating a decline in FVC of 2.6% per year [95% confidence interval (CI): (-0.031, -0.021), $p < 0.0001$; concordance correlation coefficient (CCC) = 0.92] and intermediate patients with a decline in FVC of 2.3% per year [95% CI: (-0.030, -0.015), $p < 0.0001$; CCC = 0.92]. In contrast, the relationship between age and FVC in Bethlem myopathy patients was not significant [95% CI: (-0.005, -0.001), $p = 0.1432$; CCC = 0.86] (Figure 5).

We decided to also specifically evaluate forced vital capacity measurements corresponding to ages between 5 and 15 years (5 years ≥ age ≤ 15 years; a total of 348 FVC measurements), given that this is a clinically relevant age range for decline in both respiratory and motor function. While the FVC data within each phenotypic subgroup demonstrated a pattern of continued decline (without evidence of stepwise decline), we felt that studying the rates of decline in FVC corresponding to 5-15 years of age would provide data which could be instrumental for future clinical trial planning. These subanalyses revealed that between 5 and 15 years of age FVC declined in UCMD patients by 3.5% per year [95% CI: (-0.044, -0.028), $p < 0.0001$; CCC = 0.92] whilst intermediate patients had a slightly decreased rate of decline of 1.7% per year [95%CI: (-0.031, -0.002), $p = 0.0260$; CCC = 0.91]. Again, the relationship between age and FVC in Bethlem myopathy patients was not significant [95%CI: (-0.013, 0.009), $p = 0.7261$; CCC = 0.74]. Further age stratification analyses revealed statistically significant trends in UCMD patients only, revealing a decline in FVC of 4.2% per year between 5 and 10 years of age ($p < 0.0001$), 2.9% per year between 10 and 15 years of age ($p < 0.0001$) and 2.5% per year between 15 and 20 years of age ($p = 0.0164$).

Centre	Collagen VI-Related Myopathy Patients with FVC Measurements	Males	Females	Ullrich CMD	Intermediate and Bethlem myopathy
London, UK	47	25	22	28	19
Newcastle, UK	20	10	10	3	17
Garches, France	24	14	10	14	10
Brussels, Belgium	10	5	5	7	3
Rome (B), Italy	6	2	4	3	3
Rome (C), Italy	8	6	2	2	6
Philadelphia, USA	11	7	4	11	0
Cincinnati, USA	7	4	3	1	6
Sydney, Australia	7	4	3	5	2
Melbourne, Australia	5	3	2	1	4
Totals:	145	80	65	75	70

Table 1: Demographic features of the international collagen VI-related myopathy patient cohort.

FVC = forced vital capacity; CMD = congenital muscular dystrophy; Rome (B) = Bambino Gesù Children's Hospital, Rome; Rome (C) = Catholic University, Rome

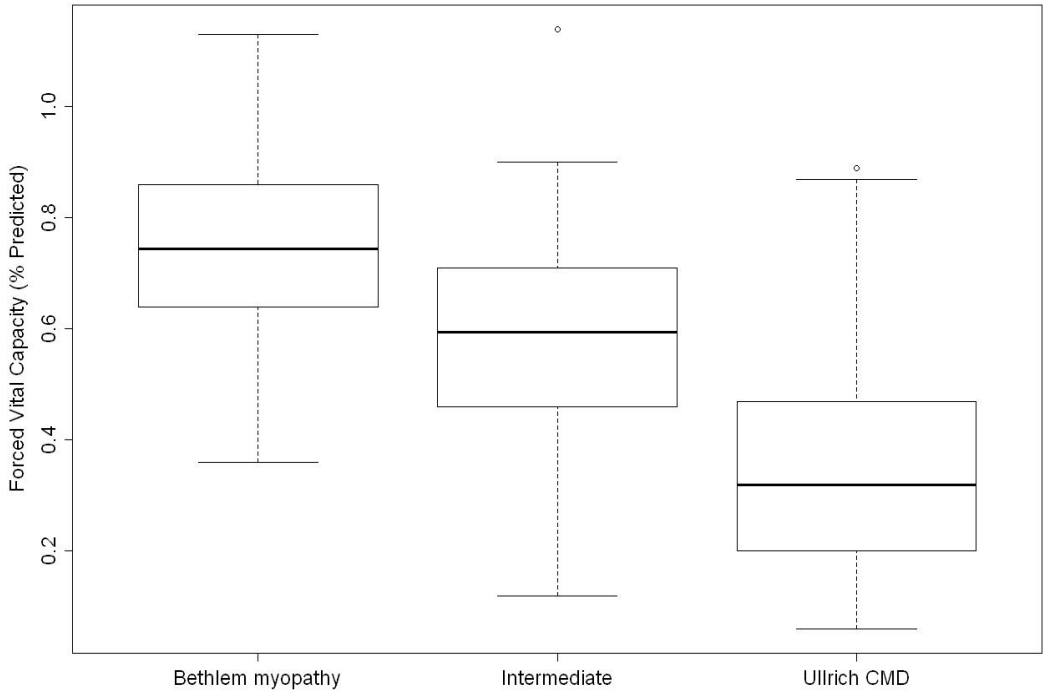


Figure 4: Box-plot demonstrating distribution of forced vital capacity measurements corresponding to the different collagen VI-related myopathy phenotypic categories.

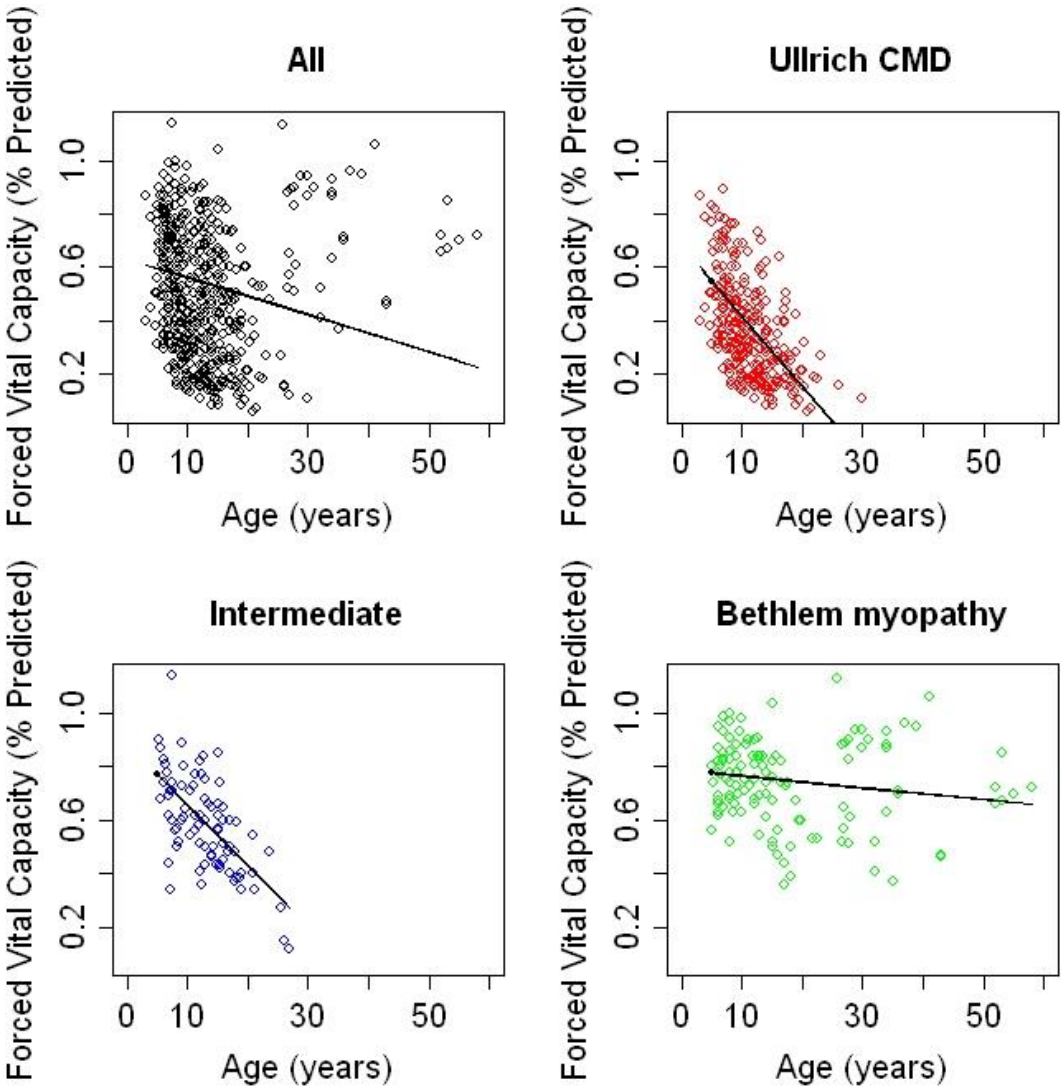
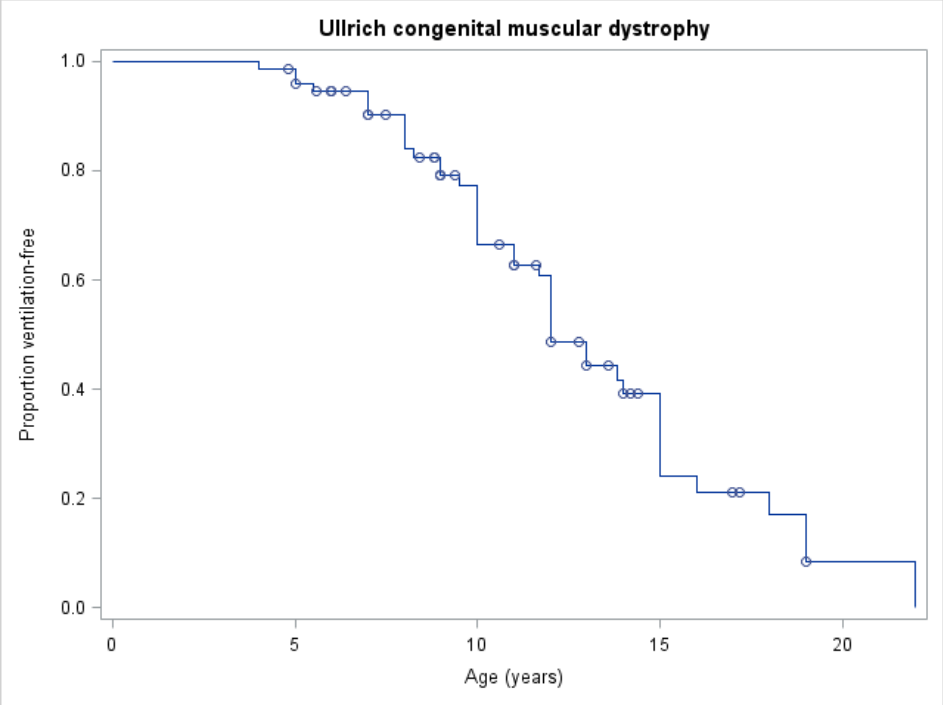


Figure 5: Profiles of decline of forced vital capacity for Ullrich congenital muscular dystrophy, intermediate collagen VI-related myopathy and Bethlem myopathy patients.

Of the 75 Ullrich CMD patients evaluated, 44 (59%) had initiated non-invasive bilevel positive pressure ventilation at the time of this study. The average age of initiation of nocturnal noninvasive ventilation (NIV) was 11.3 ± 4.0 years with an average FVC of 34% just prior to NIV initiation. Of the 27 intermediate collagen VI-related myopathy patients evaluated, 3 (11%) had started nocturnal noninvasive ventilation at an average age of 20.7 ± 1.5 years with corresponding FVC values of 41%, 50% and 60% just prior to NIV initiation. Only 1 (2%) of the 43 Bethlem myopathy patients evaluated had initiated nocturnal NIV which was at the age of 41 years. Kaplan Meier curves depicting ventilation-free probability demonstrated a statistically significant (p=0.006) difference between UCMD and intermediate patients with 50% of UCMD patients on nocturnal NIV by 11.0 years of age and 50% of intermediate patients on nocturnal NIV by 21.5 years of age (Figure 6).

Of the 486 FVC measurements analysed, corresponding maximal motor ability was available for 475 values (98%). The relationship between maximal motor ability and forced vital capacity was highly significant ($p < 0.0001$) with the distribution of FVC measurements demonstrating a direct relationship with motor ability (Figure 7). The relationship between age and FVC was highly significant within maximal motor ability categories with those patients who achieved sitting demonstrating a decline in FVC of 4.2% per year [95% CI: (-0.057, -0.027), $p < 0.0001$; CCC = 0.92], those who achieved walking with assistance demonstrating a decline in FVC of 2.1% per year [95% CI: (-0.032, -0.017), $p = 0.0003$; CCC = 0.90] and those who achieved walking independently with a decline in FVC of 0.6% per year [95% CI: (-0.009, -0.002), $p = 0.0016$; CCC = 0.95] (Figure 8). The relationship between those who achieved running and FVC was slightly significant (p= 0.0134) and demonstrated a cumulative increase in FVC of 1.2% per year [95% CI: (0.003, 0.022)]. Kaplan Meier curves depicting the probability of independent ambulation revealed that 50% of UCMD patients were non-ambulatory by 10 years of age, whilst 50% of intermediate patients were non-ambulatory by 19 years of age (Figure 9).



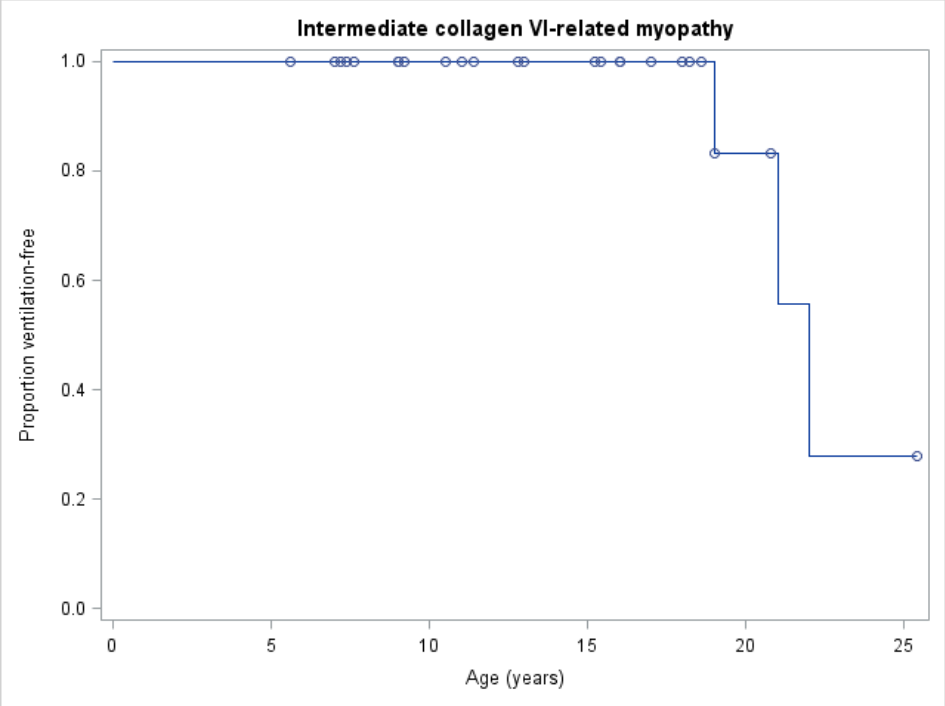


Figure 6: Kaplan-Meier curves depicting ventilation-free status in Ullrich congenital muscular dystrophy and intermediate collagen VI-related myopathy patients.

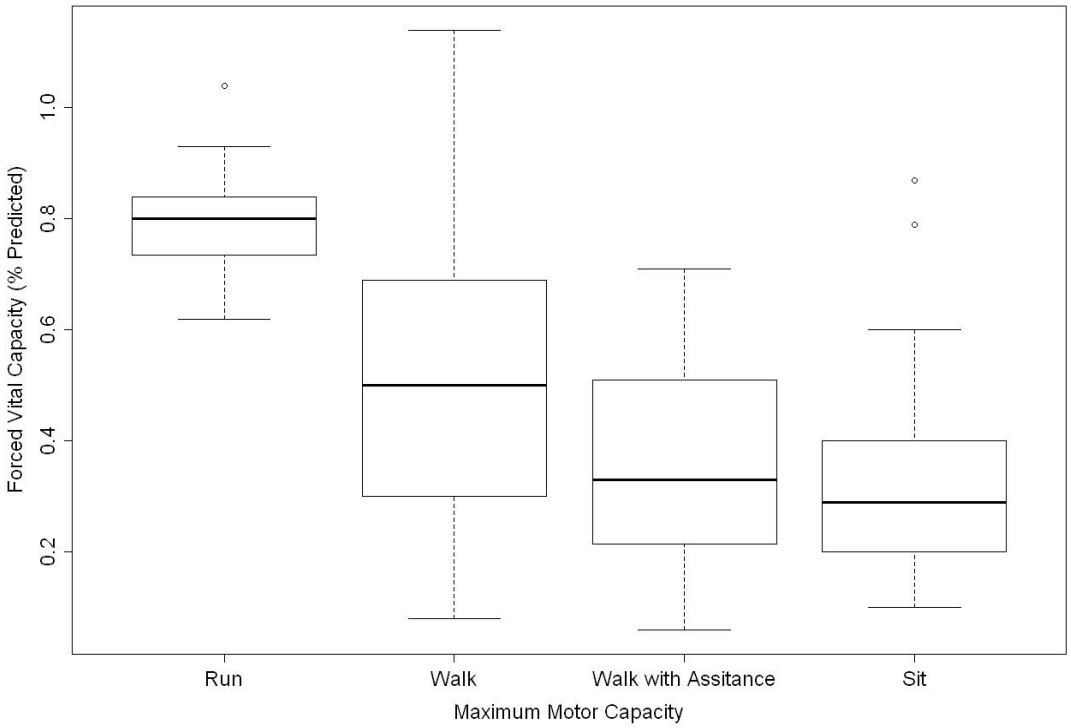


Figure 7: Box-plot demonstrating distribution of forced vital capacity measurements corresponding to different maximum motor functional abilities.

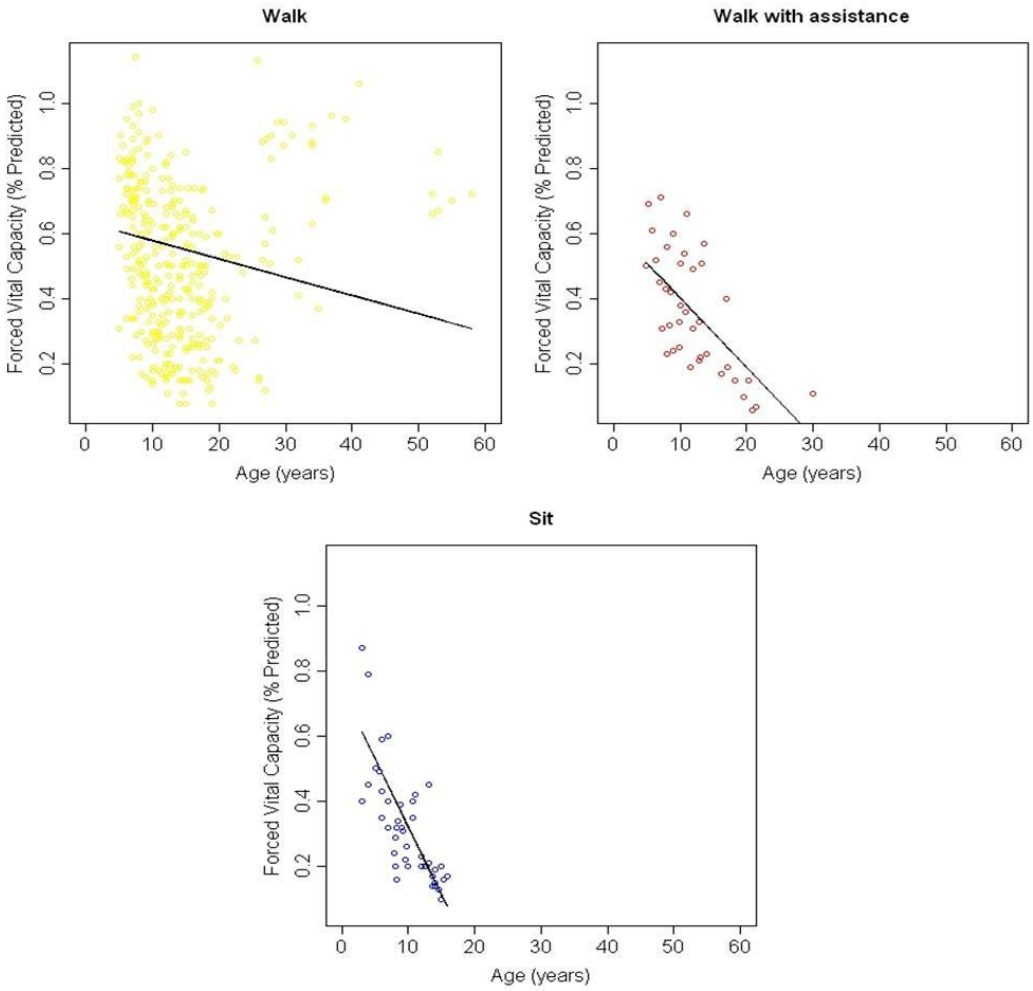
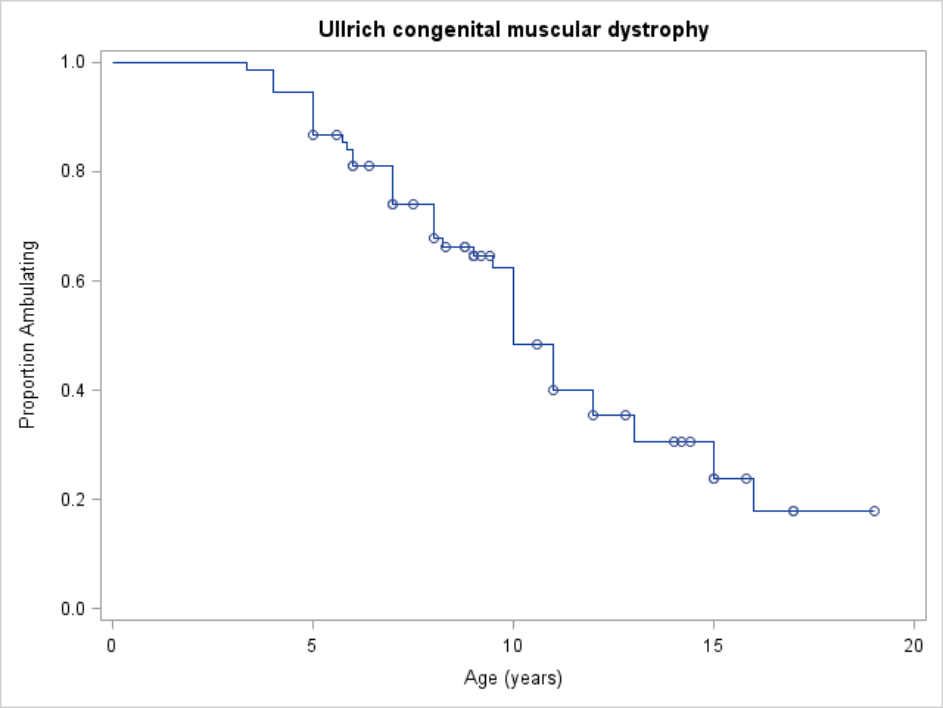


Figure 8: Profiles of decline of forced vital capacity corresponding to different maximum motor functional abilities.



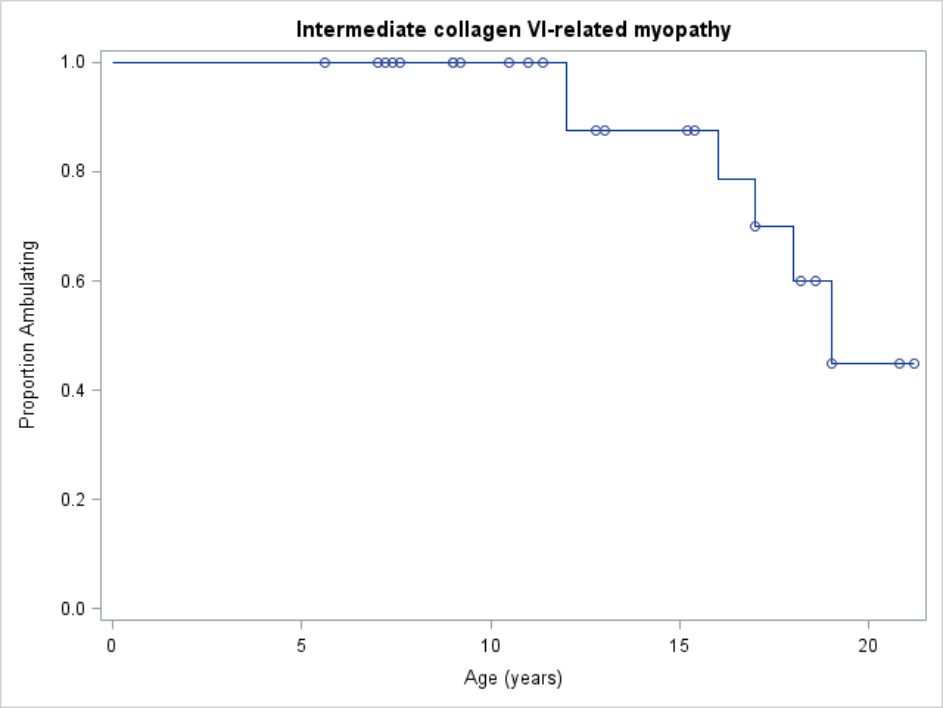


Figure 9: Kaplan-Meier curves depicting independent ambulation in Ullrich congenital muscular dystrophy and intermediate collagen VI-related myopathy patients.

2.4 DISCUSSION

In 1982, McMenamin and colleagues reported the clinical and pathologic features of 24 congenital muscular dystrophy patients. In this early, descriptive study of a cohort of CMD patients (in whom the diagnosis of CMD was based on a clinical history of congenital weakness and dystrophic muscle biopsy findings) 6 out of 24 (25%) of the patients described died of respiratory failure. Forced vital capacity measurements performed in 8 patients at a mean age of 10.75 years was less than or equal to 35% predicted.[102] In the years ensuing since that study, respiratory decompensation during childhood or adolescence, even in the setting of relatively stable muscle weakness, has become a well-recognized clinical feature of the congenital muscular dystrophies[97] and appropriate anticipation of this decompensation of great importance for clinical care.[103]

Respiratory insufficiency in muscular dystrophy patients has been attributed to a combination of weakness of inspiratory and expiratory muscles and decreased compliance of the chest wall. Conclusions from the American Thoracic Society/European Respiratory Society (ATS/ERS) Statement on Respiratory Muscle Testing include: 'respiratory muscle weakness reduces vital capacity (VC)' and 'reduction in chest wall and lung compliance, as a consequence of muscle weakness, reduces lung volumes, notably VC.'[104] Chronic breathing at lower-than-normal lung volumes is further hypothesised to be a factor in promoting chest wall stiffening which, in combination with decreased lung compliance, increases the so-called 'load' against which the respiratory 'pump' works.[105] It is this constellation of physiologic factors which results in an increased mechanical load or work of breathing on the already weakened muscles of muscular dystrophy patients.[106]

Respiratory involvement in the collagen VI-related myopathies was described in a case report of a Bethlem myopathy patient in 1999[59] and in 13 patients with Ullrich congenital muscular dystrophy in 2009.[52] A large-scale international study of pulmonary function in the collagen VI-related myopathies has not been reported to date, however. This study of longitudinal forced vital capacity measurements in 145 molecularly and/or biochemically confirmed collagen VI-related myopathy patients is the largest of its kind performed in any congenital muscular dystrophy subtype. The findings of this study increase our understanding of the natural history of this patient population in which respiratory insufficiency is a leading cause of morbidity and mortality. We have demonstrated that collagen VI-related myopathy patients at the

moderate-to-severe end of the phenotypic spectrum experience an invariable decline in pulmonary function beginning from approximately 7 years of age. In all patients whose motor function resulted in a diagnostic classification of UCMD or intermediate phenotypes, a decline in pulmonary function heralded subsequent dependence on nocturnal noninvasive bilevel positive pressure ventilation. In fact, all patients with UCMD and intermediate collagen VI-related myopathy followed this trend, making forced vital capacity a highly sensitive tool for predicting the impending risk of sleep disordered breathing/dependence on nocturnal noninvasive ventilation and respiratory failure.

This data suggests that among collagen VI-related myopathy patients, pulmonary function declines in a fashion parallel to decline in motor function/muscle strength. In particular, the observation that patients with intermediate collagen VI-related myopathy demonstrate an onset of decline in pulmonary function- and ultimate dependence on nocturnal noninvasive ventilation- later than UCMD patients follows the motor profile of these intermediate patients in whom loss of ambulation occurs in the late teenage years or young adult years, later than that seen in UCMD patients who typically lose ambulation by approximately 10 years of age (Figure 9). The parallel profiles of decline in respiratory and motor function within the UCMD phenotype and within the intermediate phenotype suggest that decline in pulmonary function in the collagen VI-related myopathies is primarily a function of muscle weakness. This assumption would be consistent with the restrictive pulmonary function pattern seen in the collagen VI-related myopathies (as well as other neuromuscular conditions) and attributed to severe weakness of inspiratory muscles.[107] Studies of pulmonary function in neuromuscular diseases have demonstrated that once respiratory muscle strength has been reduced by more than 50%, however, loss in vital capacity can be exacerbated by decreased compliance of the chest wall and the lungs.[108] Given the significant, progressive joint contractures which affect range of movement and motor abilities in collagen VI-related myopathy patients, it is possible that decreased compliance of the chest wall might be compounded by involvement of the intercostals joints. Similar to the manner in which severe contractures contribute to the loss of ambulation of UCMD and intermediate patients, it may be possible that these patients experience a more significant and consistent decline in respiratory function (compared to BM patients) due to a greater degree of decreased chest wall compliance.

This large, international study demonstrates the natural history of pulmonary function in collagen VI-related myopathies and indicates that profiles of decline in forced vital capacity in combination with respective motor function profiles can be used to stratify collagen VI-related myopathy patients into the phenotypic categories of Ullrich congenital muscular dystrophy, intermediate collagen VI-related myopathy and Bethlem myopathy (Table 2). In determining these pulmonary function profiles, FVC data gathered from the entire cohort (all ages) were included and a linear mixed model used to determine rates of decline in FVC specific to UCMD patients and intermediate patients. (Further linear mixed model analyses were performed within various age ranges, yielding age range specific rates of decline in FVC.) This proposed collagen VI-related myopathy phenotypic stratification, based on the largest study of pulmonary function in this patient population, is the first to use both motor function as well as pulmonary function criteria.

These data also demonstrate the value of profiles of decline in forced vital capacity in predicting motor function in collagen VI-related myopathy patients who remain ambulant past 10 years of age. In young collagen VI-related myopathy patients with muscle weakness either entirely preventing ambulation or enabling only assisted ambulation by the age of 7 years, a diagnosis of Ullrich congenital muscular dystrophy can be made based on motor function alone, which in turn highlights the necessity of careful surveillance of pulmonary function in these patients from an early age. In collagen VI-related myopathy patients who achieve independent ambulation, however, the phenotypic distinction between UCMD, intermediate collagen VI-related myopathy and Bethlem myopathy can challenging, particularly in young children whose future motor function can be difficult to predict or in teenagers who remain ambulant. While ambulant UCMD patients typically lose ambulation by 10 years of age and some UCMD patients never achieve ambulation, intermediate collagen VI and Bethlem myopathy patients continue ambulating, into the late teenage years and early adult years for intermediate patients and into older adult years for Bethlem patients. As this study demonstrates, FVC continues to decline in intermediate collagen VI-related myopathy patients at a rate of 2.3% per year with the average age of nocturnal noninvasive ventilation dependence of 21 years. Bethlem myopathy patients, however, do not follow a clear pattern of decline in pulmonary function over time. Although some Bethlem myopathy patients demonstrate a progressive decline

	Ullrich CMD	Intermediate	Bethlem myopathy
AMBULATION	Never walk or lose ambulation by approximately 10 years.	Ambulation lost by approximately 19 years.	Walk until adulthood. May need walking aid by 40s/50s.
PULMONARY FUNCTION	**Decline in pulmonary function is early and invariable: 2.6% per year with an average age of onset of NIV of 11 years.**	**Decline in pulmonary function starts slightly later than in Ullrich CMD but proceeds at a similar rate: 2.3% per year with an average age of onset of NIV of 21 years.**	**Decline in pulmonary function is variable and does not occur until adulthood (typically after age 40).**

CMD = congenital muscular dystrophy
NIV = noninvasive ventilation

Table 2: Phenotypic stratification of the collagen VI-related myopathies.

in pulmonary function, ultimately necessitating the initiation of nocturnal noninvasive ventilation, this study demonstrates that this is very rare and does not occur until after 40 years of age. In fact, only 1 (2%) of the 43 Bethlem myopathy patients studied in this international cohort had initiated nocturnal noninvasive ventilation, which was at the age of 41 years. Taken together, this data demonstrates the value of early motor function in the collagen VI-related myopathies in predicting profiles of pulmonary function decline as well as the value of profiles of pulmonary function decline in predicting motor function in collagen VI-related myopathy patients who remain ambulant past 10 years of age.

Given the difficulty of reliably performing pulmonary function testing in children less than 5 years of age, clinicians are naturally dependent on other clinical signs to aid in the diagnosis of Ullrich CMD. For those children not diagnosed during infancy, progressive muscle weakness and joint contractures limiting ambulation or causing a loss of ambulation by approximately 10 years help to distinguish these patients as having Ullrich CMD. As this study demonstrates, careful assessment of pulmonary function with sequential forced vital capacity measurements at least annually is essential to document the level of respiratory compromise and initiate nocturnal noninvasive ventilation for managing sleep hypoventilation. It is precisely the timely recognition and treatment of respiratory compromise in Ullrich congenital muscular dystrophy which can prevent morbidity and early mortality in this condition.

While the diagnostic distinction between intermediate collagen VI myopathy and Bethlem myopathy patients is challenging, this distinction is important for prognosticating both motor and pulmonary function in these collagen VI-related myopathy subtypes. This study demonstrates that intermediate patients demonstrate an invariable decline in forced vital capacity while Bethlem myopathy patients do not. Annual pulmonary function testing is an effective means of assessing respiratory function, which delineates the phenotypic categorisation of patients, consequently enabling the clinician to appropriately anticipate the need for non-invasive ventilation. Indeed, FVC can serve as a litmus test for predicting the likelihood of sleep hypoventilation/the need for nocturnal noninvasive ventilation. In fact, the criteria for initiating noninvasive ventilation for 'progressive neuromuscular disease' proposed by the American College of Chest Physicians is, 'maximal inspiratory pressures < 60 cm/H_2O or FVC < 50% predicted.'[109] Polysomnogram studies with either end-tidal CO_2 or transcutaneous CO_2 monitoring are recommended, as well, in order to

confirm a diagnosis of sleep hypoventilation[95] and to monitor the efficacy of noninvasive ventilation in ameliorating this sleep disordered breathing.

A recent study of 49 patients with 'early onset' collagen VI-related myopathy patients utilised motor function alone to divide patients into phenotypic categories of early severe, moderate progressive and mild. Forced vital capacity for patients categorised as 'mild' ranged from 35 - 82%[54], indicating that this category likely contains patients in the phenotypic category of intermediate collagen VI-related myopathy as well as patients in the category of Bethlem myopathy. Given that respiratory insufficiency is the leading cause of morbidity and mortality in Ullrich congenital muscular dystrophy and intermediate collagen VI-related myopathy patients, the inclusion of pulmonary function in the algorithm used to stratify collagen VI-related myopathy patients into the phenotypes of Ullrich CMD, intermediate and Bethlem myopathy is essential.

Although both sitting and supine FVC measurements are the gold standard for assessing diaphragmatic dysfunction[110] and supine FVC measurements were not routinely measured in collagen VI-related myopathy patients included in this study, it has been our experience that supine FVC values do not differ by more than 20% from sitting FVC values in this patient population. Given that a decrease in vital capacity between sitting and supine measurements of 10-30% is considered to reflect the presence of mild diaphragmatic weakness,[111] it seems likely that a degree of diaphragmatic weakness contributes to the progressive respiratory insufficiency in collagen VI-related myopathy patients. While further efforts to obtain supine FVC measurements in collagen VI-related myopathy patients will be necessary to better elucidate the degree of diaphragmatic weakness occurring in the collagen VI-related myopathies, the level of weakness can be distinguished from congenital myopathies due to *MTM1* or *MEGF10* mutations or other neuromuscular disorders including Pompe disease and spinal muscular atrophy with respiratory distress (SMARD1), for example, in which respiratory failure results from *primary* diaphragmatic dysfunction.

If indeed skeletal muscle weakness is the primary aetiology of the relentless decline in forced vital capacity observed in UCMD and intermediate collagen VI-related myopathy patients, then future therapeutic interventions aimed at slowing the progression of muscle weakness might help to reduce the progression of respiratory insufficiency in this patient population. In particular, the rate of decline in forced vital capacity could serve as an outcome measure for Ullrich CMD and intermediate

collagen VI-related patients enrolled in future clinical trials. To achieve the goal of gathering pulmonary function data over a sufficient amount of time to measure if a particular pharmacological intervention improves the natural history of decline in pulmonary function, future clinical trials in the collagen VI-related myopathy population should then endeavour to monitor forced vital capacity measurements for a minimum of 12 months' duration.

In a prospective open-label pilot trial of daily cyclosporine A performed in 6 Ullrich CMD patients between 1 to 3.2 years' duration (with patients ages at drug initiation ranging from 5.5 to 9.8 years) a decline in forced vital capacity of 7% per year was recorded.[112] This is in contrast to the rate of decline in FVC for patients found in this study, which between the ages of 5 and 15 years was 3.5% per year in Ullrich CMD patients and 2.1% per year in intermediate patients. Although this pilot trial of cyclosporine A only studied 6 UCMD patients, it is notable that the rate of decline in FVC/year indicates a more severe degree of respiratory insufficiency than on average for the UCMD patients included in this large, international study. As the natural history of the 6 UCMD patients preceding the cyclosporin A treatment is not reported, it is difficult to ascertain whether the respiratory insufficiency in the UCMD patients included in this trial was more severe than average.

While limited in size, the first clinical trial reported in Ullrich CMD patients highlights the necessity of careful natural history data and, in particular pulmonary function data, in all collagen VI-related myopathy patients considered for inclusion in future clinical trials. Furthermore, the design of future clinical trials should consider studying an age range during which time respiratory function demonstrates significant change. In particular, as the data in our study demonstrates, UCMD patients experience their steepest rate of decline in FVC (4.2% per year) between 5 and 10 years of age.

As forced vital capacity data is collected prospectively in this patient population, measures can be taken to improve both the integrity of the data collected at each neuromuscular centre as well as the consistency of data collection techniques across the various centres. The use of a standard means of measuring height in all patients is essential, as height has been found to be allometrically related to spirometric lung function.[113] An accurate measurement of height is challenging in the collagen VI-related myopathy patient population, given the potential variables of non-ambulatory status as well as progressive joint contractures and scoliosis. A standard means of

measuring height in all patients regardless of age and/or ambulatory status, such as ulnar length measured with the same equipment and technique would help to increase the accuracy of calculated percent predicted FVC values.[114] A prospective study of pulmonary function in this patient population should ideally collect FVC data in litres and apply the appropriate reference equation for calculating percent predicted FVC, using an estimation of height (ulnar length), calculated age and race.

While gathered retrospectively, this data was collected from the largest group of molecularly and/or biochemically confirmed collagen VI-related myopathy patients studied to date and provides clinical information essential for determining the natural history of the collagen VI-related myopathies. Given the size of this cohort, and the high significance of the relationship between collagen VI-related myopathy phenotypes and rates in decline in FVC, we feel that this cohort reflects the trends of both pulmonary function and motor function in individuals with collagen VI-related myopathy.

In conclusion, this large, international retrospective study demonstrates the natural history of pulmonary function in the collagen VI-related myopathies, namely that intermediate collagen VI-related myopathy and Ullrich congenital muscular dystrophy patients experience a relentless decline in forced vital capacity, culminating in nocturnal noninvasive ventilation dependence but without a progression to daytime noninvasive ventilation dependence. Furthermore, the results of this study indicate that careful and consistent monitoring of forced vital capacity is absolutely essential in collagen VI-related myopathy patients. In particular, pulmonary function testing should be performed in all patients starting by approximately age 5 years and continually at least annually. Above all, this study provides information which will result in an optimisation of respiratory surveillance, and help to decrease rates of morbidity and mortality in this patient population.

The development of potential therapies in the collagen VI-related myopathies has made possible the prospect of clinical trials, for which robust natural history data is essential for defining inclusion criteria, exclusion criteria, outcome measures, cohort sizes and trial durations. Notably, this study of the collagen VI-related myopathies, so-called ‘rare diseases,’ has resulted from an international collaboration of patients, physicians and researchers. Indeed, such collaborations have been precisely the means of driving forward natural history studies and the design and execution of clinical trials in rare diseases.[115] Rare diseases face numerous challenges on the

road toward clinical trials including that of establishing the natural history of the condition as well as achieving sufficient power in clinical trials. In designing trials for rare diseases, one option is that of using patients to serve as their own controls, which enables a trial to achieve the same power as traditionally designed trials but with fewer patients. Another approach to designing clinical trials in rare diseases includes the use of adaptive designs in which the response of early enrolled patients influences the enrolment of subsequent patients.[116] By both providing essential 'lead-in' natural history data as well as offering forced vital capacity as a relevant and viable outcome measure, this study promotes further progress toward clinical trials in the collagen VI-related myopathies.

CHAPTER 3: TOWARD AN IMPROVED DIAGNOSTIC ALGORITHM

3.1 SKIN FIBROBLAST STUDIES IN COLLAGEN VI-RELATED MYOPATHIES

The gold standard for diagnosing collagen VI-related myopathies is the sequencing of the *COL6A1*, *COL6A2* and *COL6A3* genes. This technique is complicated, however, by the large size of these genes (a combined total of 107 coding exons) and the high frequency of polymorphisms.[74] Deciding when to pursue molecular genetic screening for mutations in the collagen VI genes (*COL6A1*, COL6A2 and *COL6A3*) can be challenging, especially when assessing patients whose phenotypes fall at the milder end of the collagen VI-related myopathy spectrum, as the classic constellation of weakness, joint contractures and/or hyperlaxity seen in UCMD patients may not be clearly evident, especially in young patients. Given the time and effort involved in sequencing *COL6A1*, *COL6A2* and *COL6A3*, the challenge of interpreting variants of unknown clinical significance revealed by mutation analysis and identifying patients with collagen VI deficiency at the mild end of the phenotypic spectrum, immunocytochemical or immunofluorescence (IF) studies of collagen VI expression in fibroblast cultures has been employed by numerous neuromuscular centres as a type of 'gatekeeper' for deciding which patients should undergo *COL6A1*, *COL6A2* and *COL6A3* sequencing.

In a study of patients diagnosed with Bethlem myopathy, the muscle biopsies of five genetically confirmed BM patients were reported to have immunohistochemical findings of collagen VI-perlecan double-labelling which blinded investigators were not able to distinguish from controls. Immunocytochemical studies performed on skin fibroblast cultures, however, were interpreted by blinded investigators as demonstrating decreased collagen VI expression in 78% of genetically confirmed Bethlem myopathy patients.[117] When used prospectively to evaluate patients for a diagnosis of Bethlem myopathy, skin fibroblast immunocytochemical studies had a reported positive predictive value of 75%, a negative predictive value of 100% and a sensitivity of 100% in predicting a diagnosis of BM. The specificity of decreased expression of collagen VI in fibroblast immunocytochemical studies was notably lower at 63%. The authors suggest that immunofluorescence labelling of collagen VI using skin fibroblast cultures may avoid muscle biopsy in patients being evaluated for Bethlem myopathy and, in combination of the use of complementary DNA (cDNA) extracted from the fibroblast culture for collagen VI gene screening when indicated,

can serve as a cost effective and time effective means of evaluating patients for Bethlem myopathy.[117]

Skin fibroblast cultures are undoubtedly an important diagnostic tool for evaluating patients for collagen VI-related myopathies.[26, 68, 117-118] In skin, collagen VI is produced by skin fibroblasts, while in muscle, collagen VI is produced by muscle interstitial fibroblasts.[119] Skin fibroblast cultures facilitate the evaluation of collagen VI expression in Bethlem myopathy patients, who may have normal-appearing collagen VI expression in muscle. It is important to note, however, that some BM patients may have evidence of decreased sarcolemmal expression of collagen VI on muscle biopsy.[117]

In contrast to skin fibroblast cultures, immunohistochemical staining of skin biopsies themselves have not been found to be an effective diagnostic tool for the collagen VI related myopathies. In fact, except for completely absent or very clearly reduced collagen VI expression, skin biopsies from patients with genetically proven collagen VI-related myopathy typically demonstrated maintained collagen VI expression, even when skin fibroblast cultures revealed clearly reduced collagen VI expression.[118] The low sensitivity of skin biopsy immunohistochemical (IHC) studies for identifying underlying collagen VI deficiency is well known. In particular, in patients with collagen VI deficiency, skin biopsy IHC studies have revealed the preservation of collagen VI expression in dermal structures, including vessels, nerves, glands and smooth erector pili muscles and a decrease in the papillary dermis and surrounding hair follicles.[120-121]

Immunocytochemical studies of collagen VI expression in fibroblast cultures can reveal a complete absence of collagen VI (as in recessively inherited UCMD)[122] or evidence of abnormally formed collagen (as in autosomal dominant UCMD,[26, 122] autosomal dominant BM[117] or autosomal recessive BM[60] - Figure 10). Mutations which affect the formation of collagen VI microfibrils result in an abnormal appearance of collagen VI in skin fibroblast cultures. Terms proposed to describe the appearance of aberrantly-formed collagen VI include: 'disorganisation,' 'rarefication,' 'stippling'[117] 'dot/spot-like'[122] and 'speckled.'[60] In patients with collagen VI mutations which affect the formation of monomers, dimers and/or tetramers (and consequently the extracellular secretion of collagen VI), fibroblast IHC studies may demonstrate collagen VI expression only with permeabilisation of cells (using a detergent such as Triton X-100 or Tween 20).[117-118, 123]

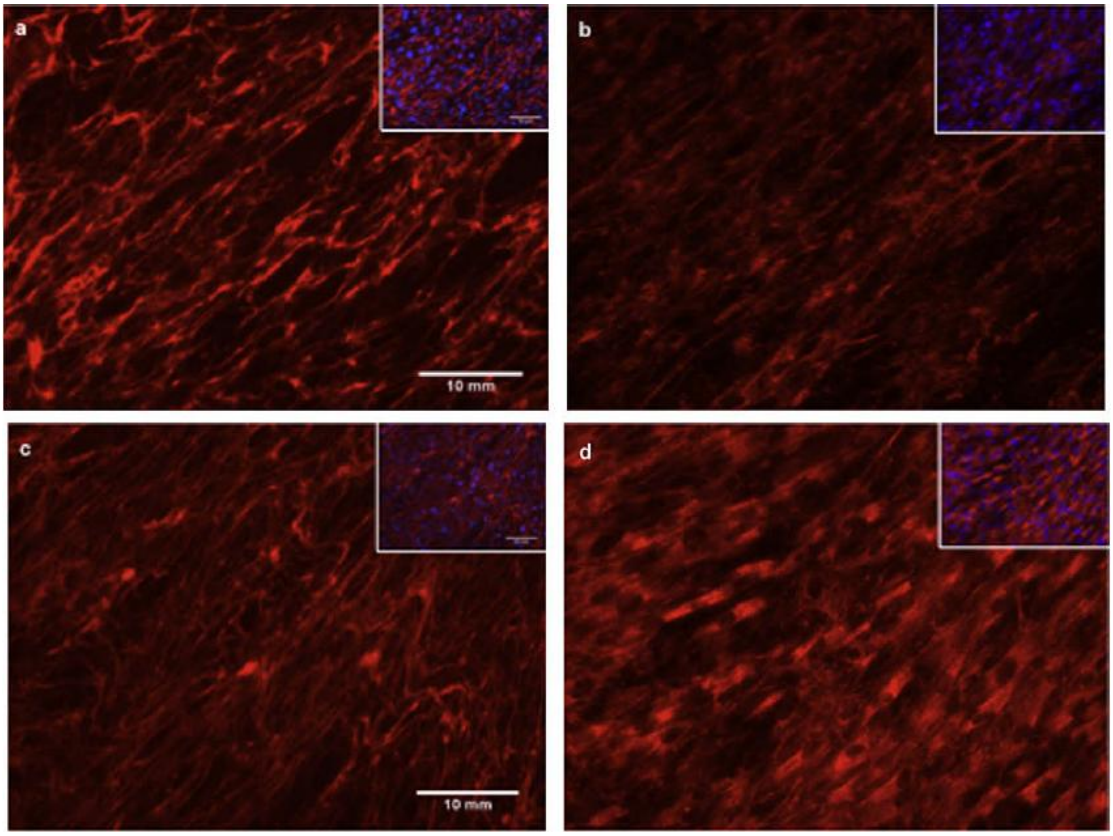


Figure 10: Skin fibroblast immunocytochemical studies.

Studies performed (by Y Hu, see Collaborators, page 5) using a monoclonal anti-collagen VI antibody in cells derived from a control patient and in a patient with autosomal recessively inherited Bethlem myopathy. Collagen VI expression in the patient (b) appears decreased compared to control (a) which has a dense-appearing and well-formed extracellular matrix. There is evidence of significant intracellular retention of collagen when the BM patient's cells are permeabilised with Triton X-100 (d) which is not seen in the control permeabilised cells (c). Insets with 4',6-diamidino-2-phenylindole (DAPI) nuclear staining. Images at x20 magnification. Measurement bar = 10 µm

(Figure from Foley et al.[60] Reproduced with permission from Elsevier.)

The conventional analysis of collagen VI immunocytochemistry in skin-derived fibroblasts is, in essence, a subjective, non-quantitative technique which, when applied in the diagnostic clinical settings, has limitations.[118] In an effort to improve our method of screening for collagen VI deficiency, our neuromuscular centre (the Dubowitz Neuromuscular Centre) investigated flow cytometry analysis as a means of quantitatively measuring collagen VI in primary skin fibroblasts and compared this method with the standard method of fibroblast collagen VI immunocytochemical analysis.

3.1.1 Materials and Methods

Eight Ullrich CMD and 5 Bethlem myopathy patients with genetically confirmed diagnoses underwent skin biopsy after informed consent was obtained. Control skin biopsies were obtained from paediatric patients without known neuromuscular disorders undergoing surgical procedures following written informed research consent.

Skin fibroblast culturing, immunostaining and flow cytometry studies were performed by J Kim (see Collaborators, page 5) and details of these techniques (as described in Kim J, Jimenez-Mallebrera C, Foley AR, et al. Flow cytometry analysis: a quantitative method for collagen VI deficiency screening. Neuromuscul Disord 2012;22:139-48[123]) are included here.

Fibroblasts were grown from skin explants and cultured in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 20% fetal bovine serum (FBS) (PAA Laboratories), 1% L-glutamine (Sigma-Aldrich) and 1% penicillin, streptomycin and neomycin (Sigma-Aldrich). Cells were cultured at 37˚C in 5% CO_2. 10^5 cells were seeded onto clean coverslips coated with fibrillar collagen I solution (PureCol 5409, Nutacon). When confluent, the medium was changed to contain 50 µg/ml of L-ascorbic acid phosphate to allow for the correct post-translational modification and secretion of collagen molecules. The medium was subsequently changed and ascorbic acid added every 2 days for 7 days' duration. Cells were then fixed with fresh 2% paraformaldehyde (pH adjusted to 7.4 ± 0.2) for 10 minutes. An aliquot of cells was permeabilised with Triton X-100 (0.05%, VWR International) in phosphate buffered saline (PBS) for 3 minutes. All cells were immunolabelled with a monoclonal primary antibody against collagen VI (MAB1944, Millipore) diluted in

PBS/Triton X-100 or PBS alone for 1 hour (1/500) at room temperature. Coverslips were washed 3 times with PBS (with or without Triton X-100), and anti-mouse biotinylated secondary antibody (1/200, Amersham) was applied for 30 minutes at room temperature which was followed by washing with streptavidin-conjugated Alexa 594 (1/1000, Invitrogen) for 15 minutes at room temperature. Nuclei were stained with Hoescht 33342 (1/2000, Molecular Probes) for 5 minutes, and preparations were examined under a Leica Digital Module R epifluorescent microscope linked to MetaMorph software (Universal Imaging).

Primary skin fibroblast cultures (standardised for passage number and at a density of $8x10^5$) were grown in 75 cm^2 tissue culture flasks (VWR International). When cells were confluent, 50 µg/ml of L-ascorbic acid phosphate was added, and cells were further incubated for 24 hours. Cells were harvested with a non-enzymatic cell dissociation solution (Sigma-Aldrich) and fixed with 2% paraformaldehyde for 10 minutes on ice. Afterwards, cells were washed with PBS (Mg^{2+} and Ca^{2+} free, Invitrogen) containing 0.1% FBS and centrifuged. Pellets were re-suspended and incubated with a monoclonal primary antibody against collagen type VI (1/250, MAB1944, Millipore) in PBS/0.1% FBS or PBS/0.05% FBS and Tween 20 (for permeabilisation) for 1 hour. For a negative control, cells were incubated without primary antibody on ice. Cells were washed twice with or without 0.05% Tween 20 and then spun down at 3000 g for 3 minutes. Rabbit anti-mouse IgG conjugated to R-Phycoerythrin (Star12A, AbD Serotec) was diluted (1/20) with either PBS/0.1% FBS or PBS/0.1% FBS and 0.05% Tween 20 for 20 minutes on ice. Cells were then washed twice with PBS (with or without Tween 20, as appropriate) and centrifuged at 3000 g for 3 minutes. Finally, cells were re-suspended in PBS/0.1% FBS and filtered through a 0.7 µm strainer to ensure cells were separated individually. Cells were then processed for flow cytometry and run on a CyAn ADP Analyzer (Beckman Coulter, California, USA) fitted with a 488 nm laser and a 633 nm red diode. Data analysis was done using Summit software (Beckman Coulter, California, USA) or Flowjo software (TreeStar, Oregon, USA). A total of 15,000 cells were analysed and gated using the two-parameter analysis side light scatter (linear scale) on the y-axis and fluorescence intensity (R-Phycoerythrin-collagen VI; log scale) on the x-axis. Side scatter is related to the cellular cytoplasmatic granularity and R-Phycoerythrin fluorescence is collected with a 575 ± 12.5 nm band pass filter (FL2 detector). A negative control (where primary antibody is omitted) was used to set up collagen VI positive immunolabelling gate, and the same gate was applied to all samples.

For assessing differences among patients groups (UCMD, BM and controls) multiple regression analysis was performed (R version 2.12.0). (Advice for the statistical analyses was obtained from Professor Timothy Cole, Department of Medical Statistics, Institute of Child Health, University College London).

My involvement in this project included (1) the collection and organisation of patient clinical information, (2) assistance with statistical analyses of the flow cytometry data, (3) interpretation of the results of the statistical analyses and their clinical relevance and (4) drafting and revision of the manuscript, which was eventually published (reference 123).

3.1.2 Results

The clinical features of the 13 patients studied are listed in Table 3. Our flow cytometry results for these 13 patients demonstrate that in non-permeabilised cells, the decrease in collagen VI surface expression in UCMD patients was statistically significant (19.6 ± 10.0) when compared with control patient cells (65.3 ± 10.7) ($p < 0.001$). The skin fibroblasts of Bethlem myopathy patients studied did not demonstrate a statistically significant decrease in collagen VI cell surface expression (54.1 ± 8.0) when compared to controls (65.3 ± 10.7) ($p < 0.1$), however (Figure 11).

When cells were permeabilised and the difference between the flow cytometry values for collagen VI expression in permeabilised and non-permeabilised cells was calculated (in order to determine the level of intracellular retention), UCMD patient cells had a statistically significant increase in intracellular retention ($70.9 \pm 7.5\%$) when compared to control ($30 \pm 10.3\%$) ($p < 0.001$). Bethlem myopathy patients also had a statistically significant increase in intracellular retention ($42.0 \pm 8.5\%$) when compared with control ($30 \pm 10.3\%$) ($p < 0.05$), albeit to a lesser degree than the UCMD patients.[123]

Clinical features and collagen VI molecular and biochemical status.

Patient	BM1	BM2	BM3	BM4	BM5	UCMD6	UCMD7
Current age (years)	47	39	†	42	7	16	21
Presentation	Birth: hip dislocation	2 years	Birth: hip dislocation	Infancy	Infancy: hypotonia and torticollis	Infancy: muscle weakness and progressive joint contractures	Birth: hip dislocation
Contractures	Elbows: + Knee: ++	Elbows: +++	Elbows: +++	Elbows:+++ Long finger flexors: +++ Achilles tendons: +++	None	Elbows: +++ Hips: +++ Knees: +++ Achilles tendons: +++	Elbows:+++ Hips:+++ Knees:+++
Maximum motor ability	Independent ambulation	Independent ambulation	Independent ambulation	Independent ambulation. Using walking frame since age 40 years	Independent ambulation	Independent ambulation. Wheelchair dependence since age 3.5 years	Independent ambulation. Wheelchair dependence since age 10 years
Respiratory function: % predicted forced vital capacity (FVC)	Not available	Not available	FVC 70% (age 23 years)	Not available	FVC 80% (age 6 years)	FVC 15% (age 9 years)	FVC 40% (age 12 years)
Mutation	*COL6A1*: intron 3 G > A + 1, causing the activation of a cryptic donor splice site in exon 3, resulting in an in-frame 66 nucleotide deletion	Heterozygous Gly341Val in exon 14 of *COL6A1*	Heterozygous mutation resulting in skipping of exon 14 of *COL6A1*	Heterozygous c.877G > A; p.Gly293Arg in exon 10 of *COL6A1*	Heterozygous c.877G > A; p.Gly293Arg in exon 10 of *COL6A1*	Heterozygous c.6210 + 1G > A in intron 16 of *COL6A3*	Homozygous c.1776 + 1G > A in intron 27 of *COL6A1*
Muscle collagen VI double immuno-labelling	Muscle not available	Muscle not available	Reduced at the sarcolemma	Muscle not available	Muscle not available	Reduced at the sarcolemma	Muscle not available
Fibroblast collagen VI immuno-labelling	Moderate reduction in the ECM; increased intracellular accumulation following permeabilisation. Overall collagen VI reduction: **	Moderate reduction in the ECM; increased intracellular accumulation following permeabilisation. Overall collagen VI reduction: **	Moderate reduction in the ECM; increased intracellular accumulation following permeabilisation. Overall collagen VI reduction: **	Moderate reduction in the ECM; increased intracellular accumulation following permeabilisation. Overall collagen VI reduction: **	Marked reduction in the ECM; marked intracellular accumulation following permeabilisation. Overall collagen VI reduction:***	Marked reduction in the ECM; marked intracellular accumulation following permeabilisation. Overall collagen VI reduction:***	Marked reduction in the ECM; marked intracellular accumulation following permeabilisation. Overall collagen VI reduction:***
Flow cytometry result•	41.2%	45.6%	28.1%	44.6%	50.5%	70.7%	60.0%

Patient	UCMD8	UCMD9	UCMD10	UCMD11	UCMD12	UCMD13
Current age (years)	19	15	30	8	23	9
Presentation	1.5 years	Birth: hip dislocation and hypotonia	1 year	1 year	Birth: talipes	Birth: talipes
Contractures	Elbows: ++ Long finger flexors: + Knees: + Achilles tendons: +++	Elbows: ++ Hips: +++ Knees: +++	Elbows:+++ Hips: ++ Knees: + Achilles tendons: +++	Elbows: ++ Knees: +	Elbows: ++ Achilles tendons: ++	Elbows: ++ Hips: + Knees: ++ Achilles tendons: ++
Maximum motor ability	Independent ambulation. Wheelchair dependence since age 11 years	Independent ambulation. Wheelchair dependence since 6 years of age	Independent ambulation. Wheelchair dependence since age 15 years	Independent ambulation. Wheelchair dependence since age 8 years	Independent ambulation. Part-time wheelchair use since age 18 years	Independent ambulation. Wheelchair dependence since age 8 years
Respiratory function: % predicted forced vital capacity (FVC)	FVC 8% (age 15 years)	FVC 16% (age 12 years)	FVC 17% (age 19 years)	Not performed	FVC 38% (age 18 years)	FVC 56% (age 7 years)
Mutation	Homozygous Cys777Arg in exon 26 of *COL6A2*	Heterozygous donor splice site change in intron 16 of *COL6A3*	Homozygous c.2839_2850del; p.Leu947_Gly950del in exon 28 of *COL6A2*	Homozygous mutation r.2839_2850del; p.Leu947Gly950del detected in exon 28 of the *COL6A2* gene	Heterozygous mutation r.868G>A; p.Gly290Arg detected in exon 10 of the *COL6A1* gene	Heterozygous mutation r.868G>C; p.Gly290Arg detected in exon 10 of the *COL6A1* gene
Muscle collagen VI double immuno-labelling	Muscle not available	Muscle not available	Reduced at the sarcolemma	Reduced at the sarcolemma	Muscle not available	Reduced at the sarcolemma
Fibroblast collagen VI immuno-labelling	Marked reduction in the ECM; marked intracellular accumulation following permeabilisation. Overall collagen VI reduction: ***	Marked reduction in the ECM; marked intracellular accumulation following permeabilisation. Overall collagen VI reduction: ***	Marked reduction in the ECM; marked intracellular accumulation following permeabilisation. Overall collagen VI reduction: ***	Marked reduction in the ECM; marked intracellular accumulation following permeabilisation. Overall collagen VI reduction: ***	Marked reduction in the ECM; marked intracellular accumulation following permeabilisation. Overall collagen VI reduction: ***	Marked reduction in the ECM; marked intracellular accumulation following permeabilisation. Overall collagen VI reduction: ***
Flow cytometry result•	68.6%	65.9%	85.8%	72.5%	74.9%	68.8%

Flow cytometry results for controls (difference between permeabilised cells and non-permeabilised cells): 19.6–42.9%.
ECM = extracellular matrix.
+ = mild contractures (5°–10°).
++ = moderate contractures (>10° but <30°).
+++ = severe contractures (>30°).
* = mild collagen VI reduction in ECM.
** = moderate collagen VI reduction in ECM.
*** = marked collagen VI reduction in ECM.
† Deceased (died of lung cancer at age 30 years).
• Difference between permeabilised cells and non-permeabilised cells (= intracellular accumulation).

Table 3: Patient clinical features.

The following patients had been previously reported: BM1[73]; UCMD6 and UCMD9[68, 74, 118]; UCMD7 and UCMD8[74, 118]; UCMD10 and UCMD12[4, 118]

(Table from Kim J et al.[123] Reproduced with permission from Elsevier.)

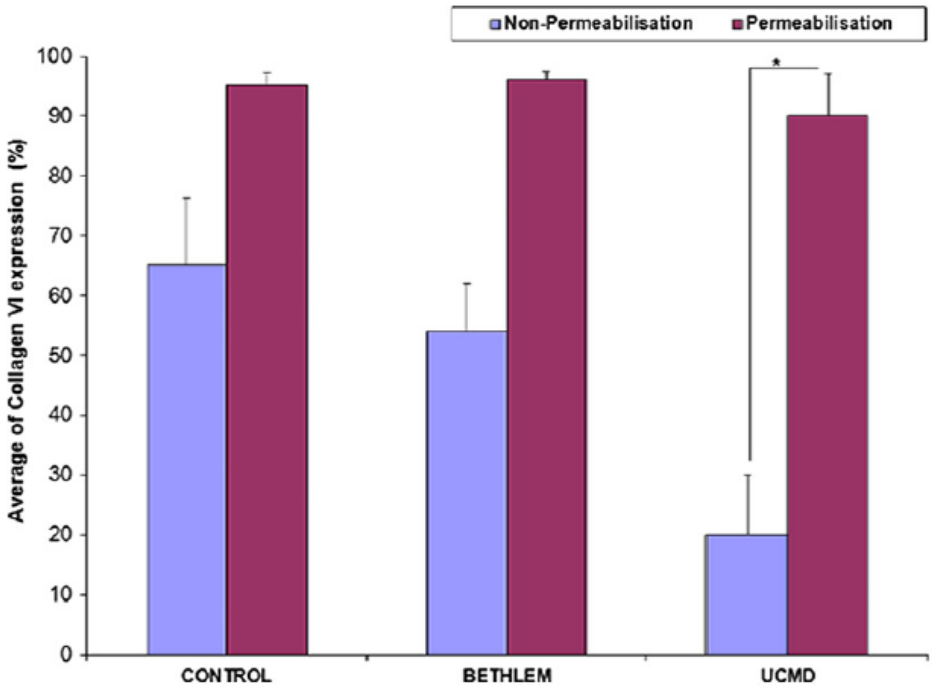


Figure 11: Quantification of collagen VI expression in skin fibroblasts using flow cytometry.

* = statistically significant (difference between collagen VI expression in UCMD patients compared to control using non-permeabilised cells).

(Figure from Kim J et al.[123] Reproduced with permission from Elsevier.)

3.1.3 Discussion

The already complicated diagnostic pathway for collagen VI-related myopathy patients is further complicated in Bethlem myopathy patients who may have a high level of residual collagen VI expression. The results of our study of the use of flow cytometry in quantitatively assessing collagen VI expression in patient fibroblast cultures indicate that this method is more objective in assessing collagen VI expression. Furthermore, technically speaking, flow cytometry is a method which is reproducible and less time-consuming than standard immunocytochemical techniques.

While flow cytometry is highly sensitive in detecting collagen VI deficiency in UCMD patients, it is less sensitive when testing fibroblasts of genetically confirmed BM patients. In fact, in some of the BM patients assessed, flow cytometry did not demonstrate a statistically significant decrease in collagen VI expression in fibroblasts compared to control fibroblasts. Given that mild Bethlem myopathy patients can have qualitative differences in collagen VI production in skin fibroblasts (as mentioned above), it is not surprising that the quantity of collagen VI may not differ significantly from controls.

Flow cytometry is an effective technique for screening for collagen VI deficiency in skin fibroblasts cultures since it is objective, rapid and reproducible. This technique may also serve as a tool for helping to determine the pathogenicity of variants of unknown significance found in *COL6A1, COL6A2* and *COL6A3* sequencing. In Bethlem myopathy patients with mutations affecting the quality of collagen VI produced in skin fibroblast cultures, however, flow cytometry may not uncover evidence of collagen VI deficiency, as the quantity of collagen VI may not significantly differ from that of controls. Given this decreased sensitivity of flow cytometry to identify Bethlem myopathy patients, the diagnostic evaluation of patients being evaluated for Bethlem myopathy should be coupled with additional studies including that of fibroblast immunolabelling techniques (focussed on the assessment of subtle quantitative and qualitative differences in collagen VI expression including the density of collagen VI, its appearance in the matrix of fibroblast cultures and the degree of intracellular retention of collagen VI in permeabilised fibroblasts) as well as muscle imaging.[123]

3.2 MUSCLE IMAGING IN COLLAGEN VI-RELATED MYOPATHIES

With the proposal of a short duration magnetic resonance imaging (MRI) protocol aimed for children with muscular dystrophy in 2002,[124] muscle MRI, once reserved for adult patients who could tolerate lying still for the duration of traditionally long MRI protocols, evolved into a valuable diagnostic tool for assessing children with muscle disease. Initial studies using MRI in primary skeletal muscle diseases demonstrated the ability of MRI (restricted to the leg) of revealing patterns of selective muscle involvement.[125] More recently, muscle MRI has been reported to detect disease-specific imaging patterns in collagen VI-related myopathies[126-128] and, in particular, with an overall sensitivity of 90% and a specificity of 96% in a cohort of patients with muscular dystrophy and spinal rigidity secondary to mutations in *COL6A1, COL6A2, COL6A3, SEPN1, LMNA* or *CAPN3.*[129]

The muscle MRI pattern seen in Bethlem myopathy patients is striking and is characterised by the involvement of the periphery with sparing of the central region of the vastus lateralis (VL) muscle (a pattern referred to as called 'outside-in') and abnormal signalling within the centre region with of the rectus femoris (RF) muscle (termed 'central shadow'[130]) (Figure 12). While this MRI pattern appears to be highly specific for Bethlem myopathy and, in fact, has been considered as practically pathognomonic for Bethlem myopathy, a study formally assessing the sensitivity and specificity of this MRI pattern for Bethlem myopathy has not yet been performed.

In a study of 19 genetically confirmed collagen VI-related myopathy patients (10 BM patients; 9 UCMD patients), 7/10 BM patients had evidence of abnormal signalling within the centre of the rectus femoris muscle. Of note, two of the BM patients without evidence of abnormal signalling in the rectus femoris were the youngest patients in the series (both 10 years of age at the time of MRI scanning). Imaging of the calves revealed a rim increased signalling between the gastrocnemius and soleus muscles in all of the BM patients except for the two patients scanned at 10 years of age. Of note, all of the BM patients had MRI evidence of abnormal signalling along the peripherally of the vastus lateralis muscle including the two patients scanned at 10 years of age.[131]

Muscle MRI in UCMD patients typically demonstrates more diffuse involvement with relative sparing of sartorius, gracilis and adductor longus muscles and often less striking vastus lateralis (VL) and rectus femoris (RF) findings. In the aforementioned

muscle MRI study in genetically confirmed BM and UCMD patients, 8/9 UCMD patients demonstrated a 'central shadow' pattern within the rectus femoris and peripheral involvement of the vastus lateralis muscle with sparing of the central region ('outside-in' pattern). Calf involvement ranged from diffuse involvement of all calf muscles (2/9) to selective involvement of the gastrocnemius and soleus muscles (7/9) and increased signal intensity between these muscles (6/9).[131]

Muscle ultrasound is another muscle imaging modality which can demonstrate disease-specific patterns. In the 1980s our neuromuscular centre (the Dubowitz Neuromuscular Centre) first described the ability of ultrasound to distinguish between normal and diseased muscles.[132] Since that time, muscle ultrasound has served as an essential diagnostic tool which can be utilised in clinic at the time of the initial evaluation of patients. An important benefit of muscle ultrasound is that it can be performed in infants and young children for whom sedation for muscle MRI may introduce undue risks.[133-136]

A report of muscle ultrasound findings in a family with Bethlem myopathy (affected mother and two daughters) demonstrated evidence of increased echogenicity within the central region of the rectus femoris muscle ('central shadow' sign) when ultrasound was performed in one of the affected daughters at 2 years and 4 months of age.[130] In a case report of a 15 year-old patient clinically diagnosed with Bethlem myopathy, muscle ultrasound of the thigh revealed clear evidence of increased echogenicity in the centre of the rectus femoris while muscle MRI performed at the same age did not real this 'central shadow' sign.[137] These findings raise the question of whether muscle ultrasound may be more sensitive than muscle MRI in detecting the 'central shadow' pattern in the rectus femoris in younger patients in whom this finding may be more subtle. In order to determine if indeed muscle ultrasound is more sensitive than muscle MRI in detecting disease-specific imaging findings in Bethlem myopathy, a formal study comparing findings from both imaging modalities performed sequentially in genetically proven BM patients of various ages and clinical severities will need to be performed.

In a study of 100 children evaluated for neuromuscular disorders using muscle ultrasound (and the criteria for grading muscle abnormalities proposed by Heckmatt[133]), muscle ultrasound was found to have a sensitivity of 78% and a specificity of 91% for detecting any type of neuromuscular disorder (including muscular dystrophies, myopathies, motor neuron disease and neuropathies). In a

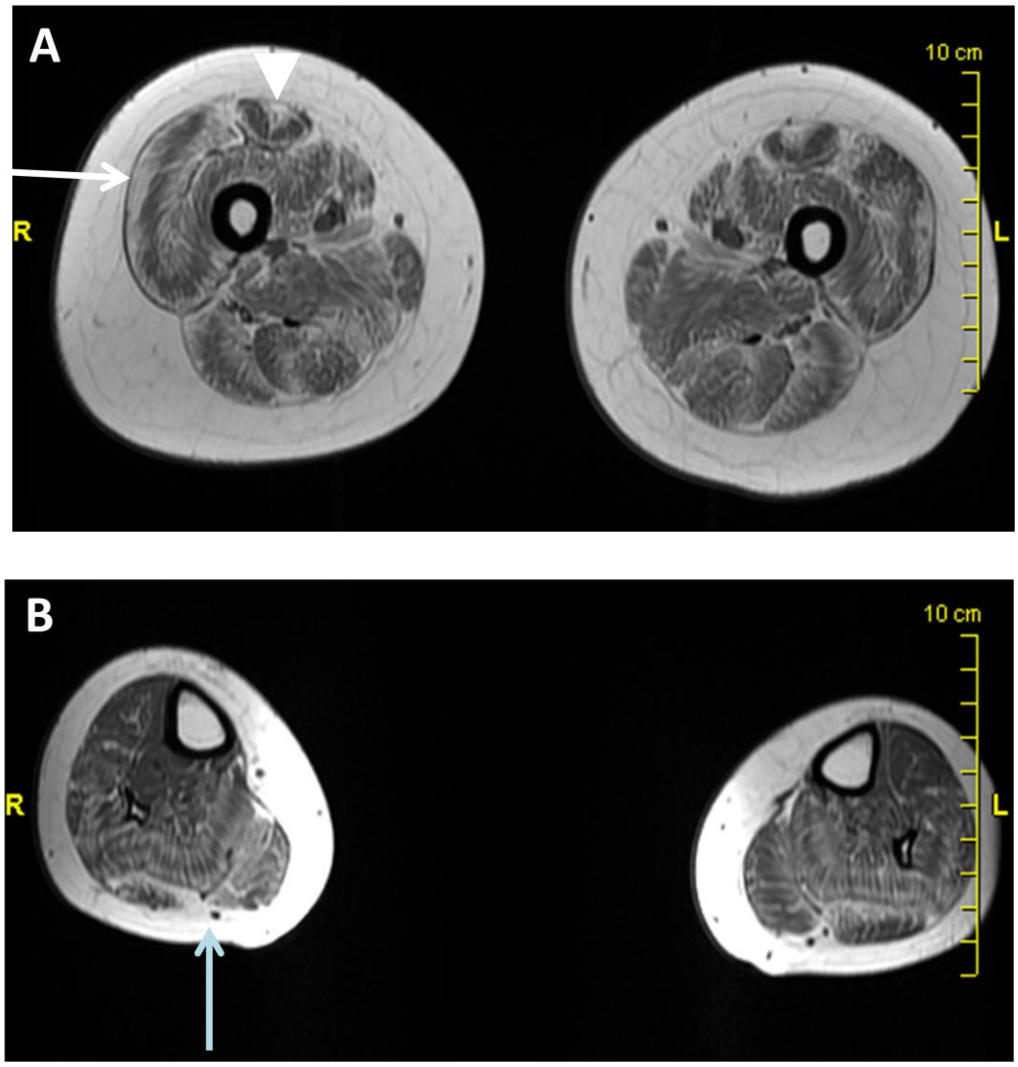


Figure 12: Muscle MRI of a Bethlem myopathy patient.

(A) T1-weighted MRI of thighs demonstrating an area of abnormal signal within the centre of the rectus femoris muscle, the so-called ‘central shadow’ pattern (arrowhead) and abnormal signal of the periphery of the vastus lateralis muscle with preservation of the interior region, the ‘outside-in’ pattern (arrow). (B) T1-weighted MRI of the calves demonstrating increased signal intensity between the gastrocnemius and soleus muscles (arrow).

retrospective study of 134 children with definitive genetic or histologic diagnoses of neuromuscular conditions, blinded evaluators correctly identified patients with muscle conditions based soley on qualitative ultrasound appearance and pattern recognition (without use of quantification of ultrasound paramaters) with an overall accuracy (mean of sensitivity and specificity) of 88%.[138] The authors of this study highlight that patterns seen with muscle ultrasonography typically are not specific for a particular neuromuscular condition except for the 'central shadow' pattern seen in Bethlem myopathy patients[138] (Figure 13).

Unlike muscle MRI, muscle ultrasound can be performed at bedside by the same clinician evaluating the patient. In this manner, muscle ultrasound becomes an extension of the neuromuscular examination, providing a non-invasive means of visualising muscles in real time, revealing findings which can be essential in narrowing the differential diagnosis and directing further diagnostic testing as well as care. Critical to the use of qualitative muscle ultrasound analyses, however, is adequate exposure of the clinician serving as the ultrasound operator/evaluator to the ultrasonographic appearances and patterns of muscle involvement evident in various neuromuscular conditions.

Undoubtedly, muscle imaging has become a crucial element of the diagnostic pathway for children being evaluated for neuromuscular disease. Furthermore, muscle imaging can help to distinguish between collagen VI-related myopathies and other conditions with similar clinical features.[139] For example, patients with *SEPN1*-related myopathy, whose spinal rigidity and respiratory involvement clinically overlap with collagen VI-related myopathy patients, have muscle imaging findings which typically reveal selective involvement of the sartorius muscle,[129, 140-141] while the sartorius is typically spared along with the gracilis and adductor longus in UCMD patients.[128] While progressive joint contractures seen in patients with *LMNA*-related Emery-Dreifuss muscular dystrophy clinically overlap with the collagen VI-related myopathies, muscle imaging findings distinguish these conditions. Prominent involvement of the posterior thigh (the vasti muscles) and more severe involvement of the medial versus the lateral head of the gastrocnemius are seen in imaging of *LMNA*-related Emery-Dreifuss muscular dystrophy patients[129, 142-143] while UCMD patients have clear sparing of the sartorius, gracilis and adductor longus muscles in the thigh and no differential involvement between the medial and lateral heads of the gastrocnemius muscle.

While the muscle imaging findings evident in the vastus lateralis and rectus femoris muscles of patients with collagen VI deficiency have been viewed as being practically pathognomonic for the collagen VI-related myopathies, the sensitivity of these findings has not been determined. Going forward, it will be essential to formally establish the sensitivity and specificity of both muscle MRI and muscle ultrasound in identifying patients with collagen VI-related myopathy. To this end, studies designed to evaluate muscle MRI and muscle ultrasound in cohorts of patients with clinical phenotypes overlapping with the collagen VI-related myopathies (including *SEPN1*-related myopathy, *LMNA*-related Emery-Dreifuss muscular dystrophy and Ehlers-Danlos syndrome (EDS) subtypes- in particular those due to mutations in *TNXB, FBN1* and *FKBP14*) will be necessary. Such studies may elucidate whether ultrasound is more sensitive than MRI in detecting collagen VI-related myopathy patients among age-matched individuals, which is an important question to address for the goal of minimising the false negative rate of muscle imaging. Additionally, such studies could help to determine the specificity of muscle MRI and muscle ultrasound for identifying collagen VI-related myopathy patients from among patients with similar clinical phenotypes.

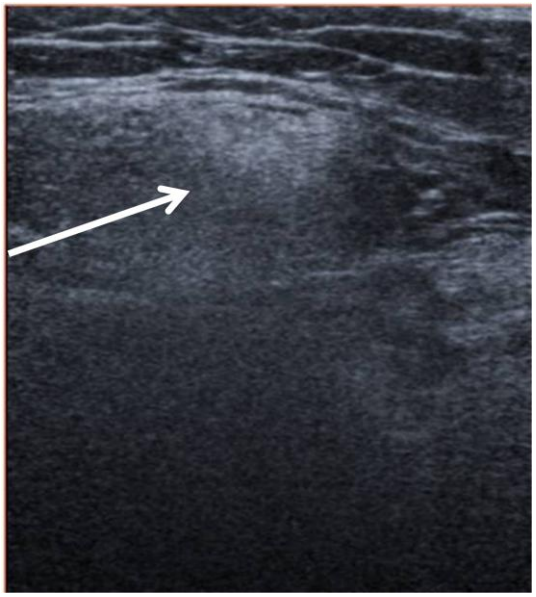


Figure 13: Muscle ultrasound of a Bethlem myopathy patient.

The highly specific ‘central shadow’ pattern is evident in the central region of the rectus femoris muscle (arrow).

3.3 PROPOSING A NEW DIAGNOSTIC ALGORITHM FOR THE COLLAGEN VI- RELATED MYOPATHIES

While the concomitant occurrence of joint contractures, distal hyperlaxity and muscle weakness is highly suggestive of a collagen VI-related myopathy, the presence of these clinical symptoms in isolation is not specific for a collagen VI-related myopathy. For example, joint contractures can be seen in other neuromuscular conditions such as Emery-Dreifuss muscular dystrophy[144] and *LMNA*-related CMD,[145] and joint hyperlaxity may be evident in numerous neuromuscular disorders.[11, 146] Further complicating matters is the neuromuscular involvement evident in patients with various primary connective tissue disorders (Ehlers-Danlos syndrome subtypes) whose distal hyperlaxity and skin findings may mimic collagen VI-related myopathies.[11, 147] Given the challenge of distinguishing collagen VI-related myopathies from clinically overlapping conditions as well the inherent complexity of arriving at a molecular or biochemical confirmation of a collagen VI-related myopathy, a clear road map or algorithm for guiding the diagnostic journey of patients being evaluated for collagen VI-related myopathy is indispensible.

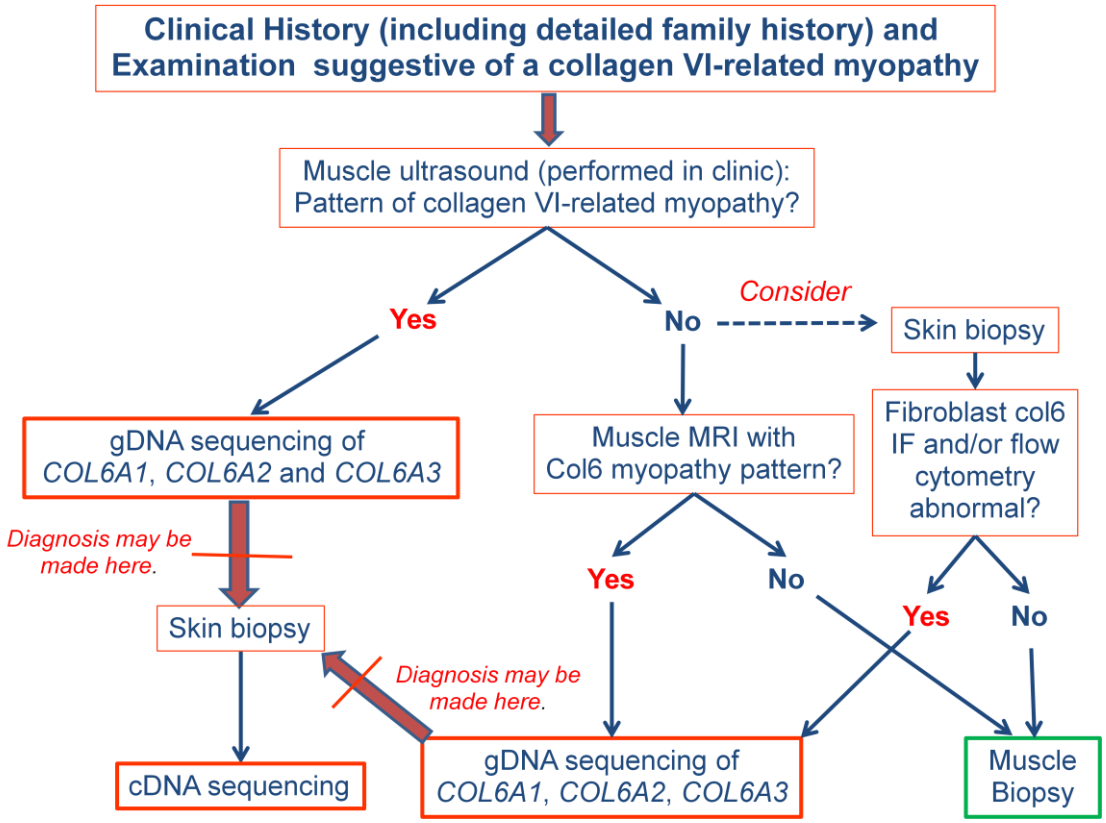
In 2001 the Dubowitz Neuromuscular Centre was commissioned as the UK Congenital Muscular Dystrophy (CMD) National Specialised Commissioning Team (NSCT) Centre with the goal of assessing, investigating and managing children with CMDs. The clinical referral criteria agreed upon by the UK CMD NSCT as the 'absolute/minimum requirements' for performing sequencing of the *COL6A1*, *COL6A2* and *COL6A3* genes are 'positive family history; reduced/absent collagen VI in muscle, abnormal collagen VI in fibroblast culture; CMD phenotype with typical concentric signal abnormality on muscle MRI.' The criteria of 'additional features' for which *COL6A1*, COL6A2 and *COL6A3* sequencing would be considered following consultation with Dubowitz Neuromuscular Centre (NSCT centre for CMDs) clinicians are: 'muscle biopsy showing dystrophic changes, offer to do collagen VI under NSCT service if muscle available; congenital/early onset hypotonia, weakness; distal laxity, proximal contractures; normal/mildly elevated CK; motor delay, walking >18 months, loss of ambulation < 20 years; progressive contractures.'[148] Using these criteria, 56% of all cases which underwent collagen VI molecular genetic screening during an 18-month period (11/2007-5/2009) had clear pathogenic mutations found in *COL6A1*, *COL6A2* or *COL6A3*, and 25% had unclassified variants. Fifty-eight percent of the

cases were sequenced using complementary DNA (cDNA) while 42% were sequenced using genomic DNA (gDNA).[149]

It is remarkable that even with a system in place for carefully selecting candidates, in whom there is high clinical suspicion for a collagen VI-related myopathy, for *COL6A1*, *COL6A2* and *COL6A3* sequencing only slightly more than half of the patients sequenced have pathogenic mutations found. At the same time, it has become more evident that muscle imaging may be more specific than collagen VI skin fibroblast immunocytochemical studies in identifying Bethlem myopathy patients. Taken together, and in an effort to improve the diagnostic journey of patients being evaluated for collagen VI-related myopathies, I have endeavoured to create a new diagnostic algorithm for evaluating patients for collagen VI-related myopathies (Figure 14).

The diagnostic algorithm I am proposing increases the emphasis placed on muscle imaging findings. This algorithm initiates with the clinical history and examination instead of with the muscle biopsy. Whilst many patients referred to our centre have already undergone a muscle biopsy, I am proposing a new approach which would not automatically necessitate a muscle biopsy. In this way, if referring centres have the ability to perform muscle ultrasound or muscle MRI and encounter findings consistent with a collagen VI-related myopathy, then gDNA sequencing of the collagen VI genes could be pursued directly. If this algorithm were adopted on a widespread basis, it may promote both the increased use of muscle imaging as well as the training of neuromuscular specialists in performing and interpreting muscle ultrasound and reading muscle MRI.

Given its portability, ease of use, ability to display muscle images in real time and, perhaps most importantly, its patient friendliness (particularly for young children), muscle ultrasound is an ideal diagnostic tool for use in the neuromuscular clinic. Furthermore, the use of muscle ultrasound to quickly visualise muscles of patients' family members (willing to undergo muscle ultrasound) may be instrumental in clarifying family history (personal experience). As significant variability in the degree of symptoms is well-known amongst patients with genetically confirmed Bethlem myopathy,[71, 130] visualisation of a 'central shadow' pattern in the rectus femoris muscle of a family member without obvious clinical symptoms would raise one's



cDNA = complementary DNA
gDNA = genomic DNA
IF = immunofluorescence
MRI = magnetic resonance imaging

Figure 14: Proposed new diagnostic algorithm for patients being evaluated for collagen VI-related myopathies.

suspicions that the family member is affected with BM. Given the apparent specificity of this muscle ultrasound pattern for BM and following my proposed diagnostic algorithm, gDNA sequencing of the collagen VI genes could be pursued directly in this situation.

In the case of patients referred to our neuromuscular centre from other centres, a muscle biopsy is typically already performed by the time the patient is clinically evaluated in our centre. In Ullrich CMD, the muscle biopsy findings of significantly decreased or absent collagen VI are typically unequivocal, allowing the diagnosis to be confirmed and appropriate as well as anticipatory care to be initiated. Intermediate collagen VI-related myopathy and Bethlem myopathy patients may have equivocal muscle biopsy findings, however. The diagnostic algorithm which I propose (Figure 14) would prompt the clinician to re-focus on the clinical history (including the family history), the examination and muscle imaging findings. In this manner, if clinical and muscle imaging findings are suggestive of a collagen VI-related myopathy, patients may proceed toward sequencing of the collagen VI genes without delay.

When variants of unknown significance are found or the effects of particular *COL6A1, COL6A2* or *COL6A3* mutations are unclear following gDNA sequencing, cDNA sequencing is recommended. A skin fibroblast culture can serve as an excellent source of cDNA. The availability of a skin biopsy also allows the opportunity for collagen VI expression studies, including flow cytometry and/or immunocytochemical studies. It is important to note that even in the setting of a clinical phenotype of a collagen VI-related myopathy and clear collagen VI deficiency on muscle biopsy, a mutation in *COL6A1, COL6A2* or *COL6A3* may not be found. Recommendations for the next step in the diagnostic journey for this particular, albeit more rare, situation are discussed in Chapter 4.

CHAPTER 4: NAVIGATING THE MOLECULAR MAZE

4.1 CHALLENGES OF GENETIC DIAGNOSIS IN COLLAGEN VI-RELATED MYOPATHIES

While a diagnosis of collagen VI-related myopathy based on clinical phenotype along with biochemical evidence of decreased collagen VI expression in muscle is sufficient for directing clinical care, arriving at a genetic diagnosis in the collagen VI-related myopathies allows patients and their families to receive accurate genetic counselling. Furthermore, a genetically confirmed diagnosis may be necessary for inclusion in future experimental clinical trials. As genetic sequencing of the collagen VI genes (*COL6A1, COL6A2* and *COL6A3*) is now available in diagnostic DNA laboratories and a clinical trial for collagen VI-related myopathy patients is presently being planned (see Chapter 6), the goal of achieving a genetically confirmed diagnosis is not only of clinical importance but also of potentially therapeutic importance.

Beyond the occurrence of both autosomal recessive and autosomal dominant inheritance, what makes the goal of arriving at a genetically confirmed diagnosis in the collagen VI-related myopathies challenging is the large number of polymorphic variants of unknown significance found in *COL6A1, COL6A2* and COL6A3, making further genetic and biochemical studies necessary in order to determine whether or not such changes are pathogenic.[51] While bioinformatics offers the potential of clarifying the effect which *COL6A1*, COL6A2 and *COL6A3* variants have on protein structure and function, such studies require time and expertise. Online databases such as the Leiden Open Variation Database (LOVD) which provides updated cataloguing of genetic variants and associated pathogenicity is a valuable resource. Another database, known as the database of Genotypes and Phenotypes (dbGAP) and developed by the National Institutes of Health (NIH), contains information regarding phenotypes associated with particular genotypes. This resource may help to improve the ease of interpretation of polymorphic variants and prevent duplicated efforts by individual laboratories in genetic, biochemical and bioinformatic type studies. Despite careful phenotyping and biochemical studies, however, there are patients with clinical and biochemical evidence of collagen VI-related myopathies in whom no pathogenic mutations in *COL6A1, COL6A2* or *COL6A3* are found.[28, 70, 150]

When clinical and biochemical evidence point toward a collagen VI-related myopathy but genetic sequencing of *COL6A1, COL6A2* and *COL6A3* does not reveal pathogenic mutation(s), the use of chromosomal microarray analyses including array comparative genome hybridization (aCGH) and single nucleotide polymorphism

(SNP)-based genomic array analysis should be considered, as conventional genetic sequencing (Sanger sequencing) does not detect the presence of copy number variations (CNVs) (which result from deletions, insertions, duplications or inversions). Both aCGH and SNP array are genetic sequencing technologies which can detect CNVs, aCGH by competitive hybridisation between patient DNA to unaffected control DNA and SNP array by hybridising patient DNA to an array designed with SNPs from multiple databases.[151]

I (along with colleagues from the Children's Hospital of Philadelphia) reported how the use of SNP array in two UCMD patients revealed evidence of large genomic deletions on chromosome 21q22.3, resulting in complete loss of *COL6A2* or both *COL6A1* and *COL6A2* on one allele in combination with either a mutation or deletion of *COL6A2* on the other allele (Figure 15). As hitherto only intragenic deletions in *COL6A1, COL6A2* or *COL6A3* had been reported,[26, 75] this was the first report of large genomic deletions as a genetic mechanism causing UCMD.[152]

In particular, the phenotypes of two of the patients reported [patient 1 (P1) and patient 2 (P2)] were consistent with Ullrich CMD, and their parents had no history of neuromuscular disease and normal neuromuscular examinations. Patient 2's brother had epilepsy and global developmental delay but no evidence of neuromuscular symptoms, as would be seen in a congenital muscular dystrophy. Patient 3 (P3) had a SNP array performed due to symptoms of global developmental delay and hypotonia. His phenotype was not consistent with a collagen VI-related myopathy. Patient 3's father had a normal neuromuscular examination, and muscle ultrasounds performed both in P3 and his father were normal in appearance.

Genomic DNA sequencing in P1 revealed a heterozygous intronic nucleotide change (G>A) at position c.1970-9 at the intron 25-exon 26 junction of *COL6A*2 (Figure 15). Using cDNA extracted from P1's fibroblast, *COL6A*2 sequencing revealed a 7 base pair insertion resulting from the use of a novel splice acceptor site in intron 25, created by the mutation (Figure 15). This insertion causes a frameshift and results in a premature stop codon (G656AfsX17). SNP analysis in P1 revealed a 69 kilobase (kb) genomic deletion at 21q22.3, encompassing at least 18 exons of *COL6A*2 (Figure 15). P1's asymptomatic mother was found to be heterozygous for the *COL6A*2 nucleotide change (G>A) at c.1970-9, and his father was found to be heterozygous for the 69 kb deletion. SNP-based genome array analysis in P2 revealed 2 deletions at 21q22.3: a 1.61 Megabase (Mb) deletion encompassing the

entire *COL6A1* and *COL6A*2 genes as well as surrounding genes on one allele and a smaller deletion of 47 kb encompassing the entire *COL6A*2 gene on the other allele (Figure 15). DNA sequencing of P2's parents revealed that P2's mother was heterozygous for the 1.61 Mb deletion and his father was heterozygous for the 47kb deletion. Neither parent had mutations in *COL6A1* or *COL6A*2 in the nondeleted alleles. P2's brother was found to be heterozygous for the 1.61 Mb deletion encompassing *COL6A1* and *COL6A*2. SNP array performed in P3 due to a history of developmental delays and hypotonia revealed a heterozygous 1.09 Mb deletion encompassing *COL6A1* and *COL6A*2 as well as adjacent genes (Figure 15). P3's asymptomatic father was found to carry the same 1.09 Mb deletion.

The finding of these large genomic deletions on chromosome 21 involving *COL6A1* and *COL6A2* established a new type of mutational mechanism in the collagen VI-related myopathies. This finding also adds to the complexity of genetic evaluations in the collagen VI-related myopathies, as large genomic deletions would not be detected by single-exon amplification and sequencing (unless performed quantitatively). Furthermore, hemizygous changes occurring on the nondeleted allele will seem to occur in apparent homozygosity, thus potentially obscuring the true genetic causation of the patient's condition.

The observation made that the parents of patients 1, 2 and 3 are clinically asymptomatic despite carrying deletions encompassing *COL6A2* or both *COL6A1* and *COL6A2* provides the most conclusive evidence to date that haploinsufficiency for collagen VI is not a disease mechanism for collagen VI-related myopathy, and in particular for Bethlem myopathy- as had been hypothesised in the literature.[153] This finding is of great translational importance since it indicates that potential therapeutic strategies which are aimed at eliminating a dominant-negatively acting mutation- and would result in a functional state of haploinsufficiency- would not result in clinical symptoms of neuromuscular disease.

Finally, these findings indicate the importance of considering the use of chromosomal microarray analyses (including aCGH and SNP array genomic analyses) in the diagnostic evaluation of patients with phenotypes suggestive of collagen VI-related myopathies. In particular, chromosomal microarray analysis should be considered in

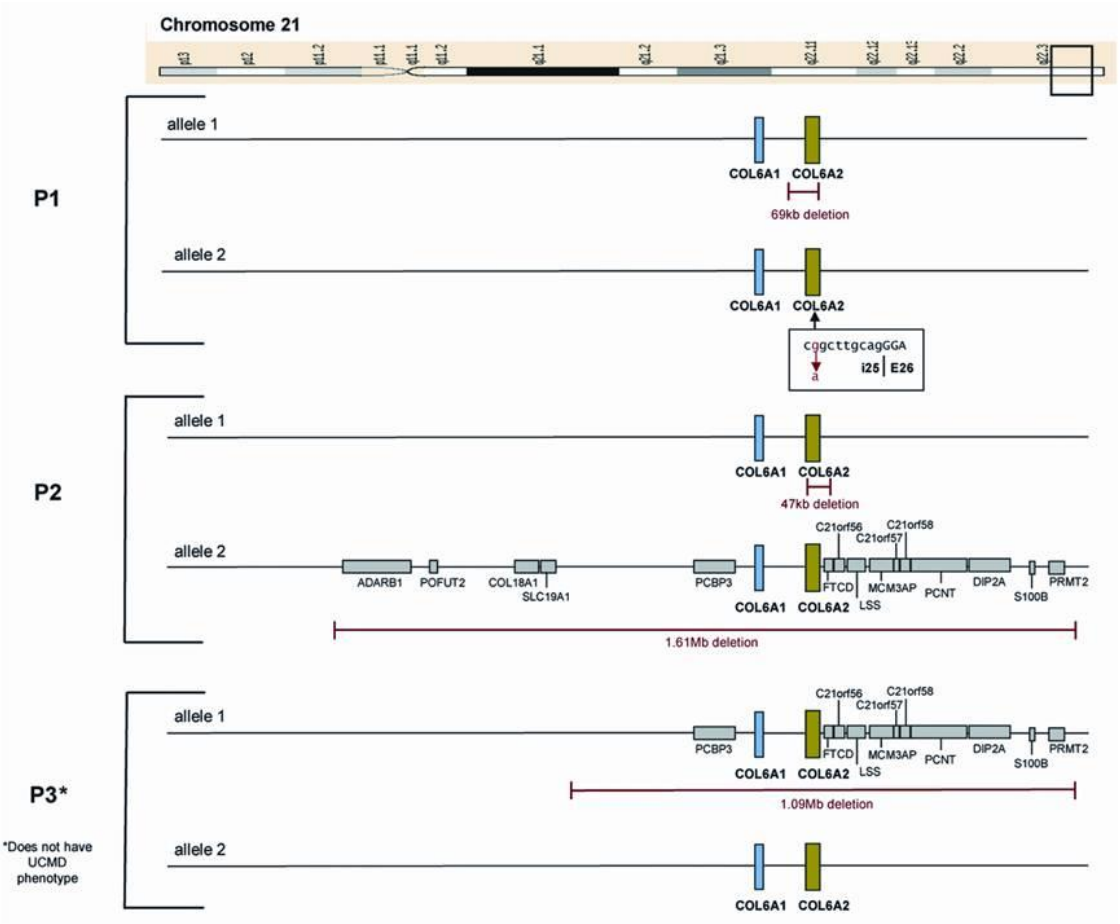


Figure 15: Mapping of genomic deletions on chromosome 21q22.3.

(Chromosome 21 ideogram from http://www.genecards.org.)

(Figure from Foley et al.[152] Reproduced with permission from John Wiley and Sons.)

patients with convincing collagen VI-related myopathy phenotypes in whom no mutations are identified in *COL6A1, COL6A2* or *COL6A3.* Furthermore, in those patients in whom a heterozygous mutation found in *COL6A1, COL6A2* or *COL6A3* cannot account for the patient's phenotype, chromosomal microarray analysis should be considered, especially if this mutation is carried in an asymptomatic parent. When a mutation in *COL6A1, COL6A2* or *COL6A3* on one allele occurs in combination with a large genomic deletion encompassing an entire collagen VI gene (*COL6A1, COL6A2* or *COL6A3*) on the other allele, the mutation found with conventional Sanger sequencing will appear to occur in homozygosity; therefore, the presence of apparently homozygous mutations in *COL6A1, COL6A2* or *COL6* in patients without a family history of consanguinity should prompt chromosomal microarray analysis, as well.

4.2 THE ROLE OF NEXT GENERATION SEQUENCING IN THE EVALUATION OF COLLAGEN VI-RELATED MYOPATHIES

For those patients who demonstrate a classic collagen VI-related myopathy clinical phenotype and muscle biopsy findings of collagen VI deficiency, in whom no pathogenic mutation is identified by either gDNA or cDNA sequencing of *COL6A1, COL6A2* and *COL6A3*, a yet undiscovered causative gene may be responsible for the clinical and biochemical manifestations of collagen VI deficiency. New genetic technologies including 'next-generation sequencing' (NGS) or 'high-throughput sequencing' offer the potential of discovering further causative genes for collagen VI deficiency. In contrast to conventional sequencing methods ('first generation' or 'low-throughput' sequencing), which require polymerase chain reaction (PCR) amplification of all exons and adjacent intron regions and Sanger sequencing of the genes, high-throughput sequencing allows massively parallel sequencing, simultaneously sequencing thousands of genes at one time.

The increased potential to uncover new causative genes through the use of high-throughput sequencing offers an unprecedented opportunity for potentially determining the molecular causation in patients with suggestive clinical phenotypes and biochemical evidence of collagen VI deficiency who have been hitherto molecularly uncharacterised. The discovery of new causative genes in the collagen

VI-related myopathies could further our understanding of pathways involved in collagen VI deficiency with promising therapeutic implications.

4.2.1 Whole-genome sequencing

Whole-genome sequencing is a form of high-throughput sequencing which allows sequencing of the entire genome (chromosomal DNA as well as mitochondrial DNA) at one time. A major advantage of this sequencing technology is its ability to sequence a large number of genes remarkably faster and at significantly lower cost than traditional low-throughput methods. In particular, the cost of whole-genome sequencing fell from 100,000,000 US dollars in 2001 to below 10,000 US dollars in 2011 (National Human Genome Research Institute).[154]

The advantages of whole-genome sequencing over the conventional, targeted, exon-capture approaches in genetically heterogeneous Mendelian disorders have been well-recognised.[155] In whole-genome sequencing, however, individual sequence reads are smaller and of lower accuracy than traditional Sanger sequencing, factors which may affect the ability to 'capture' or 'call' variants which may be of clinical significance.[156] Furthermore, given the immense quantity of data produced by whole-genome sequencing, a limiting factor to its use is (1.) the amount of time necessary for wading through the data and (2.) clinical as well as bioinformatics expertise for recognising relevant variants from among the many hundreds of variants reported.

The extension of the use of whole-genome sequencing to commercial genome sequencing companies has made possible the independent access of patients to whole-genome sequencing. While the role of whole-genome sequencing in the diagnostic evaluation of patients with phenotypes suggestive of collagen VI-related myopathies has not been formally studied, there are limitations to this sequencing technology. In particular, some of these limitations have been demonstrated in the case of two Bethlem myopathy patients who pursued commercial genome sequencing.

The proband presented to the Neuromuscular Clinic (National Hospital for Neurology and Neurosurgery, NHNN) at 33 years of age. He was never able to run, and he had developed slowly progressive proximal weakness beginning in his teenage years. Examination revealed bilateral elbow contractures, Achilles tendon tightness and

proximal muscle weakness [Medical Research Council (MRC)-grade 4/5]. Deep tendon reflexes were present. Creatine kinase was 354 IU (reference range: <150 IU). The patient's mother reported a similar history, albeit with a later onset with proximal muscle weakness noted around age 30 and progressing to dependence on a cane for walking by age 60 years. Muscle biopsies performed in the proband and his mother revealed dystrophic-appearing muscle. No other family members were noted to have joint contractures and/or muscle weakness. The family was counselled that their condition was likely a form of autosomal dominant muscular dystrophy with Bethlem myopathy and Emery-Dreifuss muscular dystrophy included in the differential diagnosis. Further investigations were recommended; however, the family did not return to the NHNN for the ensuing 5 years.

When the family was reassessed in 2010, they had an electronic storage device with their annotated whole-genome sequence data (performed commercially by a human genome sequencing company). Analysis of the kindred genome data revealed several variants carried by the proband and his mother (but no unaffected family member) in genes implicated in neuromuscular diseases. The strongest candidate shared by both patients was a heterozygous missense variant (G347A; p.Ser116Asn in exon 3 of *COL6A1)* which is modestly common (~8% allele frequency) in the general population. Given the finding of this *COL6A1* variant, I became involved in reviewing this case with colleagues at the NHNN (both genetically and clinically). We decided that the interpretation of these uncertain genomic variants necessitated further elucidation of the patients' pattern of muscle involvement and recommended lower extremity muscle MRI be performed.

MRI in the proband and his mother revealed abnormal signalling in the central region of the rectus femoris muscle ('central shadow' pattern) and abnormal signalling along the periphery of the vastus lateralis muscle ('outside-in' pattern) (Figure 16). Given this muscle MRI pattern strongly evocative of Bethlem myopathy and consistent with our clinical impression, we pursued *COL6A1, COL6A2* and *COL6A3* sequencing in a diagnostic laboratory (Guy's and St Thomas' DNA Laboratory, London). Genomic DNA sequencing revealed a heterozygous c.6365G>T (p.Gly2122Val) mutation in exon 20 of *COL6A3* in both the proband and his mother. Skin biopsies performed in both the proband and his mother for the purpose of collagen VI immunocytochemistry studies in dermal fibroblasts revealed abnormal collagen VI expression. In re-analysing the whole-genome sequencing data it was evident that

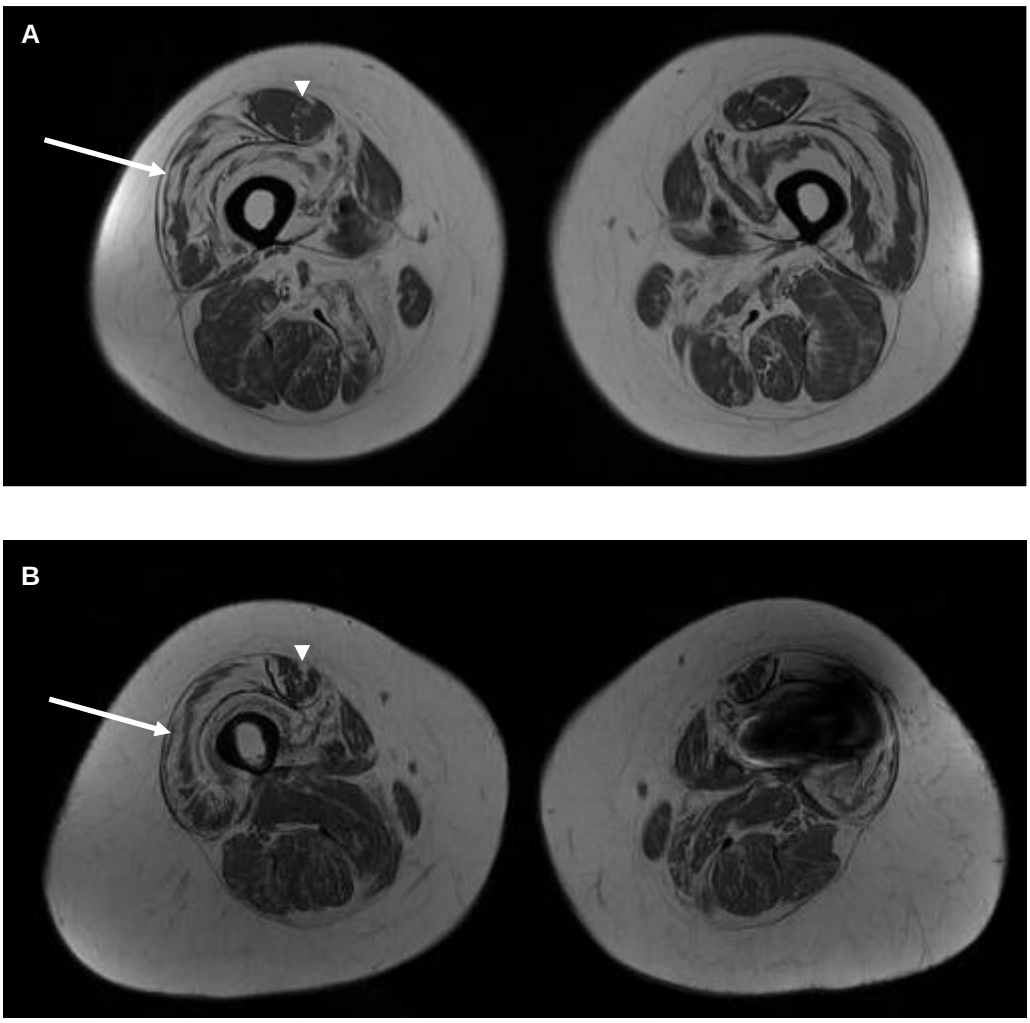


Figure 16: Muscle MRI demonstrating patterns specific for Bethlem myopathy.

Axial cut through mid-thigh region of the proband (A) and his mother (B). Abnormal signalling in the central region of the rectus femoris muscle ('central shadow' pattern) (arrowheads). Abnormal signalling along the periphery of the vastus lateralis muscle ('outside-in' pattern) (arrows). (Signal artefact in left leg of the proband's mother (B) due to metallic implant.)

this *COL6A3* mutation had not been 'called' or identified as a variant of potential pathogenic significance.

One potential factor leading to the inability of whole-genome sequencing to identify this pathogenic *COL6A3* mutation may be the guanine-cytosine (GC)-rich content of the collagen VI genes.[12, 157] High GC content has been identified as a factor associated with poor capture rates using high-throughput sequencing technologies.[158-160] Given that GC pairs are bound by three hydrogen bonds (versus the two hydrogen bonds binding adenine-thymine pairs), DNA with higher GC content is more stable, making it more difficult to anneal during the PCR amplification process. To improve 'coverage' (percentage of genome sequenced) of GC-rich areas, amplification-free high-throughput sequencing techniques have been trialled in order to decrease the amplification 'bias' of PCR for GC-rich areas of DNA.[161-162] While high-throughput sequencing protocols are being modified to accommodate the issue of poor coverage over GC-rich areas, statistical methods of estimation and correction for this issue of GC content bias are still being understood.[163]

Another potential reason why the pathogenic *COL6A3* mutation was not identified by whole-genome sequencing may be related to the information available on the coding transcripts of *COL6A3* in the genetic and bioinformatic databases utilised in the analyses of the whole-genome data, for predicting functional effects of particular variants. In contrast to locus-specific databases, such as Leiden Open Variation Database (LOVD), the generalised tools used for interpreting variants in whole-genome sequencing do not have the benefit of detailed information of variants nor precise phenotypic correlations for variants such as available in LOVD.

As this case clearly highlights, the role of the clinician is essential in directing the diagnostic pathway for collagen VI-related myopathy patients. In this instance, the patients independently pursued whole-genome sequencing, the findings of which served to distract the diagnostic journey from its original course. When, however, the patients returned to the recommended diagnostic pathway (following the collagen VI-related myopathy diagnostic algorithm – see Figure 14) and muscle MRI was performed, imaging findings highly specific for Bethlem myopathy were evident (Figure 16). These findings prompted Sanger sequencing of the collagen VI genes and lead to the discovery of the pathogenic *COL6A3* mutation in both the proband and his mother, thereby providing genetic confirmation of their Bethlem myopathy diagnosis.

4.2.2 Exome sequencing

Exome sequencing involves sequencing of all protein-coding exons - estimated at approximately 200,000. By interrogating the 1.5% of the genome containing an estimated 95% of pathogenic variants, exome sequencing greatly expedites efforts to identify new genes as well as causative mutations in known genes in Mendelian disorders.[164-178] What remains to be seen, however, is if this high-throughput sequencing technology will be able to move from the research realm, where it plays an important role in gene discovery, to the clinical setting, where it may ultimately serve as a diagnostic tool.[159]

Our neuromuscular centre (the Dubowitz Neuromuscular Centre) has had the opportunity to collaborate with the Wellcome Trust Sanger Institute on a large-scale exome sequencing research project called the UK10K project. As part of UK10K Rare Diseases Subgroup, we have had the opportunity to submit DNA samples for exome sequencing at the Sanger Institute. During the past two years I have coordinated a cohort of 125 neuromuscular patients from our neuromuscular centre who have conditions without a previously identified genetic aetiology. I collected detailed phenotypic data, gathered DNA samples and prepared these samples for submission to the Sanger Institute, where exome sequencing is performed.

While exome sequencing in all samples submitted as part of the UK10K project has not yet been completed, the results of those samples which have exome sequencing and exome data analyses completed have demonstrated the power of this high-throughput genetic technology in uncovering the genetic aetiology in hitherto molecularly uncharacterised neuromuscular patients. Our experience has demonstrated that the approach of studying families with multiple affected members sharing distinct or ‘deep’ phenotypes has proven to be a powerful method for reducing a pool of candidate genes in rare, monogenic disorders. In particular, patients with genetically uncharacterised congenital muscular dystrophies are ideal candidates for exome sequencing, particularly given their typically autosomal recessive inheritance, the benefit of biobank material (muscle biopsies/myoblast cultures and skin biopsies/fibroblast cultures) and extensive patient phenotyping including muscle imaging (MRI and ultrasound) which our neuromuscular centre has available for these patients.

Indeed, the careful selection of affected individuals from informative families (for which family history is well known as well as DNA from affected and unaffected individuals available) has been crucial for gene discovery in rare diseases.[179-180] In particular, sequencing patients with autosomal recessively inherited conditions of deep clinical phenotype has been a particularly successful method of finding causative variants in hitherto molecularly uncharacterised patients, even when sequencing a single affected individual.[181]

In particular, the exome sequencing performed through our neuromuscular centre's collaboration with the Sanger Institute has identified 5 novel genes to date. Three of these genes (*ISPD*, *β3GALNT2* and *GMPPB*) were found to be causative of dystroglycanopathy phenotypes while 2 of these genes (*DYNC1H1* and *DCTN2)* were found to be causative of distal spinal muscular atrophy (dSMA) phenotypes. Work on these genes is in progress at present with manuscripts describing the characterisation of these genes underway. While I have dedicated a considerable effort to the exome sequencing project over the course of the past two years, this work was performed as part of a larger collaborative initiative with the Sanger Institute, and I have elected to concentrate the scope of my thesis on the collagen VI-related myopathies, for which my input has been that of a lead investigator.

The collagen VI-related myopathies are genetically heterogeneous, given that some patients with phenotypes consistent with collagen VI-related myopathies and with clearly decreased or absent collagen VI expression in muscle have no evidence of mutations in the known collagen VI genes (*COL6A1, COL6A2* and *COL6A3*). Based on the success of exome sequencing in uncovering novel causative genes in autosomal recessively inherited conditions, it seems probable that exome sequencing may uncover new causative genes in molecularly uncharacterised patients with clinical and muscle biopsy evidence suggestive of collagen VI deficiency.

In filtering large volumes of sequencing data produced by exome sequencing in patients with clear autosomal recessive inheritance, variants occurring in heterozygosity are excluded. The caveat when studying collagen VI-related myopathy patients is that *de novo* autosomal dominant inheritance has been recognised as a common mutational mechanism in the known collagen VI genes (*COL6A1, COL6A2* and *COL6A3*) (section 1.4.1). When the possibility of pathogenicity of heterozygous variants (such as in *de novo* autosomal dominant

inheritance) exists, the task of studying/filtering large quantities of exome sequencing data is more challenging. In the setting of suspected *de novo* autosomal dominant inheritance, exome sequencing of parents is essential. Submission of parental DNA along with proband DNA as so-called 'trios' is an exome sequencing strategy with has proven to be successful in identifying novel variants in known genes.[182-184]

4.2.3 Discussion

While our current understanding of variations in non-coding regions of the genome is limited, mutations in these regions may be disease causing. For this reason, and given the potential of the price of whole-genome sequencing to continue to fall, whole-genome sequencing may start to be preferred over exome sequencing as a more time and cost-effective diagnostic tool.[180] At present, however, technological challenges exist for both whole-genome and exome sequencing technologies which need to be overcome for high-throughput sequencing techniques to become viable as clinical diagnostic tools. For either whole-genome or exome sequencing to be considered as tools for routine clinical use, high accuracy will be essential.[185] There is a need for automated algorithms for annotating variants as well as improved means of determining the functional impact of rare or novel variants.[186] Furthermore, standards and guidelines for testing and reporting of whole-genome and exome sequencing data will need to be established prior to these high-throughput sequencing technologies being offered by clinical laboratories as clinical tests.[187]

Analogous to the need for an algorithm to arrive at a clinical and biochemical diagnosis of collagen VI-related myopathy, there is a need for an algorithm to ensure an accurate molecular diagnosis, as well. The starting point of a molecular diagnostic algorithm for collagen VI-related myopathies would be with conventional (Sanger) sequencing of the collagen VI genes (*COL6A1, COL6A2* and *COL6A3*) performed on genomic DNA (gDNA). Most commonly, the initial clinical evaluation of patients begins with a series of blood tests including creatine kinase. For this reason, it is common for gDNA to be extracted from blood drawn at the time of initial evaluation. This gDNA can then be Sanger sequenced for *COL6A1, COL6A2* and *COL6A3.*

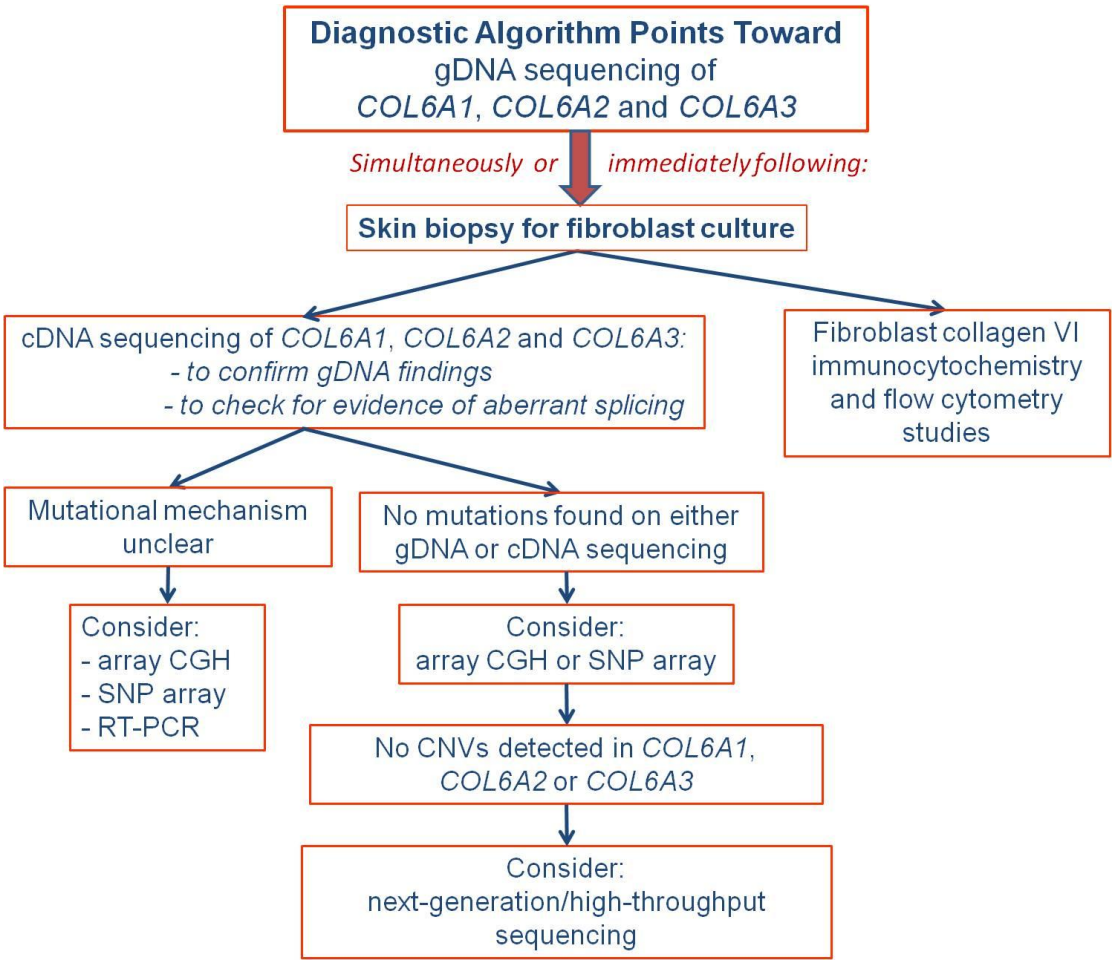
Given the risk of Sanger sequencing of missing heterozygous large deletions or duplications or deep intronic mutations affecting splicing, cDNA sequencing should be considered, particularly if no clear pathogenic mutation has been identified by

gDNA sequencing. cDNA sequencing can identify some deletions and duplications and exon skipping events resulting from splicing mutations. Indeed, this later mutational mechanism is common in the collagen VI-related myopathies (section 1.4.1). Since cDNA is synthesised from mRNA, effects of alternative splicing are evident. Furthermore, the incomplete use of an alternative splice site can be revealed by reverse transcriptase polymerase chain reaction (RT-PCR) evidence of different sized bands. Fibroblast cultures grown from skin biopsies are an ideal source of cDNA. As cDNA sequencing plays an important role in complementing gDNA sequencing results, a skin biopsy is recommended for patients being evaluated for collagen VI-related myopathies. Furthermore, skin biopsy serves as a source of fibroblasts for use in both collagen VI immunocytochemical studies as well as flow cytometry studies, which are important components of the collagen VI-related myopathy diagnostic algorithm (Chapter 3).

As Sanger sequencing does not identify copy number variations (CNVs) resulting from large deletions, insertions, duplications or inversions, the use of chromosomal microarrays (array CGH or SNP array) should be considered in patients with clinical phenotypes and muscle biopsies studies convincing for collagen VI deficiency in whom mutations in *COL6A1, COL6A2* or *COL6A3* have not been identified (section 4.1). It is important to point out that at the present time neither whole-genome sequencing nor exome sequencing technologies are able to consistently identify copy number variations, although this shortcoming is starting to be addressed. For this reason, it seems logical to pursue chromosomal microarray analysis before embarking on next-generation/high-throughput sequencing.

The increased potential of finding causative mutations in known or novel genes in genetically uncharacterised collagen VI-related myopathies patients made possible by next generation sequencing is unprecedented. While the advent of the era of genomic medicine is undoubtedly an exciting time, it is important that genetic evaluations in patients evaluated for collagen VI-related myopathies be pursued in a coordinated and careful manner. To this aim, I am proposing an algorithm for genetic analyses in the collagen VI-related myopathies (Figure 17). As this proposed algorithm indicates, next-generation/high-throughput sequencing should be reserved for patients in whom careful gDNA and cDNA sequencing of *COL6A1, COL6A2* and *COL6A3* have been performed as well as chromosomal microarray. This algorithm demonstrates that skin biopsy serves a dual role as a source of cDNA as well as a means for skin fibroblast immunocytochemistry and flow cytometry studies, which

serve to complement the ongoing genetic studies being performed in patients evaluated for collagen VI-related myopathies. Indeed, it was by following this genetic analyses algorithm (Figure 17) that the adult Bethlem myopathy patients described above (section 4.2.1), who pursued commercial whole-genome sequencing, arrived at a genetic confirmation of their diagnosis when gDNA sequencing revealed a causative mutation in the *COL6A3* gene.



cDNA = complementary DNA
CGH = comparative genome hybridisation
CNVs = copy number variations
gDNA = genomic DNA
RT-PCR = reverse transcriptase polymerase chain reaction
SNP = single nucleotide polymorphism

Figure 17: Algorithm for genetic analyses in the evaluation of patients for collagen VI-related myopathies.

CHAPTER 5: HISTOLOGICAL AND IMMUNOHISTOCHEMICAL FINDINGS IN COLLAGEN VI DEFICIENT MUSCLE

5.1 HISTOLOGICAL FINDINGS IN COLLAGEN VI-RELATED MYOPATHIES

The interpretation of muscle biopsies in the collagen VI-related myopathies can be challenging. Unlike the congenital myopathies which are characterised by particular structural abnormalities such as nemaline rods, cores, cap-like structure, etc, specific morphological abnormalities are not seen on histological studies of muscle in patients with collagen VI-deficiency. In fact, the muscle pathology is often not overtly 'dystrophic' in the collagen VI-related myopathies. Dystrophic muscle is typically considered to show evidence of necrosis and regeneration;[188] however, necrosis frequently is not seen in collagen VI deficiency. On haematoxylin and eosin (H&E) staining necrosis is visualised in fibres which stain palely and frequently are filled with phagocytes. Necrotic fibres also stain positive for acid phosphatase stain.[188]

Muscle fibre regeneration can be more difficult to determine. It can be evident by basophilic fibres, which on H&E staining appear to have a blue-coloured cytoplasm related to high RNA content. Vesicular nuclei (nuclei which are small, rounded and with a transparent nucleoplasm and prominent nucleoli) associated with basophilic fibres are felt to represent regeneration, as well.[188] Small, basophilic fibres in muscle biopsies of collagen VI-related myopathy patients likely represent regenerating fibres; however, small non-basophilic fibres could represent atrophic fibres or fibres which have not matured. Fibres in muscle biopsies of collagen VI deficient patients older than 6 months of age which stain positive for fetal myosin indicate pathologically immature fibres and are typically reflective of fibre regeneration, but fetal myosin can also stain nuclear clumps (darkly-stained nuclei which are shrunken in appearance and frequently occur in small groups in chronic muscular dystrophies or denervation[188]). Fibres which stain with both fetal myosin and developmental myosin, however, typically represent regenerating fibres.

Other histopathology features observed in muscular dystrophies including variation in fibre size, whorled fibres, split fibres, fibrosis and increased adipose tissue can be evident in muscle biopsies of collagen VI-related myopathy patients. In an early paper about a condition labelled 'Ullrich's disease' (as the categorisation of this condition as a 'muscular dystrophy' had not been clearly established), a histological and histochemical analysis of 5 muscle biopsies from patients clinically diagnosed with 'Ullrich's disease,' reported variation in fibre size, internal nuclei and fibrosis in

all 5 biopsies with evidence of type I fibre predominance in 3 of the biopsies. Evidence of necrotic fibres was observed in only 2 of the 5 biopsies studied, however.[48] Another study of muscle histopathology characteristics of two UCMD patients (one molecularly confirmed) reported evidence of neonatal myosin positive fibres only in the smallest fibres of the biopsies and a striking absence of developmental myosin staining on those fibres. The authors of this study hypothesise that these findings may represent a problem with muscle fibre regeneration or maturation evident in collagen VI-related myopathies.[189] A further histopathology study of molecularly confirmed UCMD patients revealed that early biopsies performed in UCMD patients (performed between 6 and 30 months of age) revealed evidence of fibre atrophy. The authors propose that UCMD may be a primary atrophic myopathy rather than a primary dystrophic myopathy.[190] The authors further state that this hypothesis would be consistent with the finding of normal to only mildly elevated CK values in UCMD patients.[190] Indeed, unlike most CMDs including merosin-deficient CMD and the dystroglycanopathies in which CK elevation is pronounced, collagen VI-related myopathy patients have normal to mildly elevated CK levels.[5, 191]

Histopathology findings may offer clues as to underlying pathophysiological mechanisms in the collagen VI-related myopathies. In particular, if collagen VI deficiency results in deficient or abnormal muscle fibre regeneration, research strategies focussed on muscle regeneration- such as with satellite cells- may hold therapeutic promise for collagen VI deficiency. Lack of cardiac involvement in collagen VI-related myopathy patients may suggest aberrant regeneration as a potential pathophysiological mechanism, particularly given the fact that skeletal muscle regeneration is mediated by satellite cells[192-193] while cardiac myocytes lack this regenerative capacity given their absence of satellite cells or analogous type of precursor cells.[194-195]

The occurrence of cores in the muscle biopsies of patients with collagen VI-related myopathy has been noted in the cohort of patients evaluated at our centre (Dubowitz Neuromuscular Centre);[188] however, the frequency of this histopathology finding in the collagen VI-related myopathy patient population had not been studied. Cores have been classically associated with the 'core myopathies' resulting from mutations in *RYR1* or *SEPN1.* Cores are not specific to the *RYR1* or *SEPN1*-related core myopathies, however, and have been described in several conditions including neurogenic conditions and myopathies with a variety of genetic causes.

Ultrastructurally, cores are characterised by a decrease or an absence of mitochondria and a variable degree of Z-line streaming and myofibrillar disorganisation. Minicores are characterised ultrastructurally by focal myofibrillar disorganisation and smearing of the Z-line.[188]

For the purposes of diagnosis, however, it is the finding of reduced or absent collagen VI on immunohistochemical (IHC) studies, more than any particular histopathology finding in patients with collagen VI deficiency, which can effectively direct the diagnostic work-up towards sequencing of *COL6A1*, *COL6A2* and *COL6A3*. At times, however, basal lamina defects can be subtle in muscle biopsies of collagen VI-related myopathy patients, which can cause the interpretation of perlecan-collagen VI IHC double-labelling to be challenging. The decision to proceed with muscle IHC studies (collagen VI-perlecan double-labelling) is often based on muscle histological findings in combination with available clinical information. In this scenario, nonspecific muscle histological findings could result in a collagen VI-related myopathy patient's diagnosis being delayed or even missed. The aforementioned histopathology study of early muscle biopsies in molecularly confirmed UCMD patients highlighted the presence of fibre atrophy and fibre-type disproportion.[190] Indeed, the muscle histopathology findings of patients with collagen VI deficiency who are less than one year of age are highly suggestive of a myopathy. It is precisely in these young children whose muscle biopsies may demonstrate nonspecific myopathic findings where the greatest challenge lies in identifying candidates for collagen VI immunohistochemical studies. Delaying or missing a diagnosis of collagen VI-related myopathy could result in increased morbidity and possibly mortality in UCMD and intermediate collagen VI-related myopathy patients whose relentlessly decline in pulmonary function must be recognised and addressed in a timely manner (see Chapter 2).

5.1.1 Materials and Methods

I performed a comprehensive retrospective review of muscle biopsies in 15 collagen VI-related myopathy patients, as well as muscle biopsies of 7 *RYR1* and 7 *SEPN1*-related core myopathy patients, given the histopathology overlap of these conditions. I reviewed the clinical notes of the patients whose biopsies were assessed in order to ensure that only cases with molecularly confirmed diagnoses (with proven mutations

in *COL6A1, COL6A2* or *COL63*) were included. Biopsies were carefully reviewed by myself as well as by a neuropathologist (Dr Rahul Phadke), who was blinded to the biopsy findings originally reported by another pathologist (Professor Caroline Sewry). Dr. Phadke and I together assessed each muscle biopsy for the presence of internal/central nuclei, regeneration and necrosis, excessive connective tissue, fat, structural abnormalities, fibre typing and fibres staining positive for fetal myosin. The general pattern of a biopsy was labelled 'dystrophic' if there was evidence of both necrosis and regeneration. If necrosis and regeneration were not seen in the biopsy, but other findings such as increased internal/central nuclei and/or structural abnormalities were present, the biopsy was categorised as 'myopathic.'

5.1.2 Results

Cores and/or minicores were present in the muscle biopsies of 12/15 (80%) of molecularly confirmed collagen VI-related myopathy patients studied (Table 4). Both large and small fibres positive for fetal myosin were present in biopsies of collagen VI-myopathy patients in contrast to the presence of only very small (<5 microns) fetal myosin positive fibres typically seen in the muscle biopsies of *RYR1*-related core myopathy patients. *SEPN1*-related myopathy muscle biopsies demonstrated patterns of fetal myosin positive fibres mimicking that of collagen VI-related myopathy muscle biopsies, however.

The appearance of cores and minicores seen in collagen VI-related myopathy patients was similar to the cores and minicores seen in the biopsies of patients with *RYR1*-related myopathy or *SEPN1*-related myopathy (Figure 18). While 8/15 (53%) of the collagen VI-related myopathy patient biopsies assessed were categorised as having a dystrophic appearance (based on evidence of necrosis and regeneration), none of the *RYR1*-related myopathy patient biopsies assessed and only 1/7 (14%) of *SEPN1*-related myopathy patient biopsies assessed was found to be dystrophic-appearing. Of note, while 2 *RYR1*-related myopathy biopsies had evidence of regenerating fibres, there was no evidence of necrosis to fulfil the criteria necessary for being considered dystrophic.

Patient Number	Gender, age (years) at time of biopsy, phenotype	General pattern	Internal/ central nuclei	Regeneration and Necrosis	Excess Connective Tissue	Fat	Structural Abnormalities	Frequency of Structural Abnormalities	Fibre typing	Fetal Myosin
Collagen VI-Related Myopathy Patients										
1	F, 1, UCMD	dystrophic	few	present	none	none	none	none	2 fibre type pattern	small and large
2	M, 2.2, UCMD	Myopathic	few	absent	endomysial, mild, focal	absent	cores + minicores	patchy	2 fibre type pattern	not assessed
3	M, 2.3, UCMD	dystrophic	few	present	endomysial, mild, diffuse	peri- and endomysial, moderate, patchy	cores	focal	2 fibre type pattern	not assessed
4	M, 2.5, UCMD	dystrophic	few	present	endomysial, mild, diffuse	peri- and endomysial, moderate, patchy	cores + unevenness	patchy	2 fibre type pattern	very small, small and large
5	F, 3, UCMD	dystrophic	few	present	endomysial, mild, diffuse	peri- and endomysial, moderate, patchy	cores + unevenness	patchy	2 fibre type pattern	very small, small and large
6	F, 3.2, Int	dystrophic	few	present	none	none	cores + unevenness	focal	2 fibre type pattern	very small, small and large
7	M, 4, Int	dystrophic	frequent	present	endomysial, mild, diffuse	endomysial, mild, patchy	cores + minicores	patchy	2 fibre type pattern	very small, small and large
8	M, 4, BM	dystrophic	few	present	endomysial, mild, focal	endomysial, moderate, patchy	cores + unevenness	patchy	2 fibre type pattern	small and large
9	F, 4, UCMD	Myopathic		absent	peri- and endomysial	peri- and endomysial	none	none	2 fibre type pattern	small and large
10	M, 4.8, UCMD	Myopathic	frequent	absent	peri- and endomysial, marked, diffuse	peri- and endomysial, marked, diffuse	cores + unevenness	patchy	2 fibre type pattern	small and large
11	M, 4.8, UCMD	dystrophic	frequent	present	peri- and endomysial, moderate, diffuse	peri- and endomysial, marked, diffuse	unevenness	patchy	2 fibre type pattern	not assessed

Patient Number	Gender, age (years) at time of biopsy	General pattern	Internal/ central nuclei	Regeneration and Necrosis	Excess Connective Tissue	Fat	Structural Abnormalities	Frequency of Structural Abnormalities	Fibre typing	Fetal Myosin
12	M, 7.3, UCMD	Myopathic	frequent	absent	peri- and endomysial, marked, diffuse	peri- and endomysial, moderate, patchy	cores + unevenness	focal	2 fibre type pattern	very small, small and large
13	F, 11.8, UCMD	Myopathic	few	absent	peri- and endomysial, moderate, diffuse	perimysial, mild, focal	cores + unevenness	patchy	2 fibre type pattern	small and large
14	M, 14.5, Int	Myopathic	few	absent	peri- and endomysial, moderate, patchy	endomysial, marked, diffuse	cores + unevenness	patchy	uniformity	not assessed
15	F, 18.6, UCMD	Myopathic	few	absent	peri- and endomysial, mild, diffuse	peri- and endomysial, marked, diffuse	cores + minicores + unevenness	patchy	2 fibre type pattern	very small, small and large
***RYR1*-Related Core Myopathy Patients**										
16	M, 1	Myopathic	frequent	present (2 regenerating fibres)	peri- and endomysial, mild, diffuse	peri- and endomysial, moderate, patchy	none	none	uniformity	very small and small
17	F, 2.7	Myopathic	occasional	absent	none	none	cores + minicores	widespread	type 1 predominance	not assessed
18	F, 3.4	Myopathic	occasional	absent	endomysial, mild focal	perimysial, moderate diffuse; endomysial, mild, focal	cores	widespread	type 1 predominance	very small
19	M, 4.8	Myopathic	frequent	absent	none	none	cores	widespread	type 1 predominance	very small
20	F, 9.8	Myopathic	few	absent	endomysial, mild focal	perimysial, marked, diffuse	cores + minicores	patchy	type 1 predominance	very small
21	F, 12.4	Myopathic	occasional	absent	none	none	cores	focal	type 1 predominance	very small and small
22	F, 15.3	Myopathic	very frequent	present (one regenerating fibre)	endomysial, mild, focal	perimysial, marked, diffuse	cores + unevenness	widespread	2 fibre type pattern	very small, small and large

Patient Number	Gender, age (years) at time of biopsy	General pattern	Internal/ central nuclei	Regeneration and Necrosis	Excess Connective Tissue	Fat	Structural Abnormalities	Frequency of Structural Abnormalities	Fibre typing	Fetal Myosin
***SEPN1*-Related Core Myopathy Patients**										
23	M, 2	Myopathic	occasional	absent	none	none	minicores	focal	2 fibre type pattern	none
24	F, 2.3	Myopathic	occasional	absent	none	none	cores + minicores	patchy	2 fibre type pattern	very small
25	M, 2.5	Myopathic	occasional	absent	none	none	cores + minicores	focal	2 fibre type pattern	very small and large
26	F, 4	Myopathic	occasional	absent	perimysial, mild, diffuse	peri- and endomysial, moderate, patchy	cores + minicores + unevenness	patchy	2 fibre type pattern	very small, small and large
27	M, 11.9	Myopathic	occasional	absent	endomysial, mild, focal	perimysial, marked, diffuse	cores + minicores	focal	2 fibre type pattern	very small, small and large
28	F, 12.6	Myopathic	occasional	absent	none	perimysial, mild, focal	minicores	widespread	2 fibre type pattern	very small
29	M, 37.3	dystrophic	few	present	endomysial, mild, focal	perimysial, marked, diffuse	cores + minicores + unevenness	patchy	2 fibre type pattern	very small and large

BM = Bethlem myopathy
F = female
Int = Intermediate
M = male
UCMD = Ullrich congenital muscular dystrophy

Table 4: Histopathology features of collagen VI-related myopathy muscle biopsies compared to *RYR1*-related core myopathy and *SEPN1*-related core myopathy muscle biopsies.

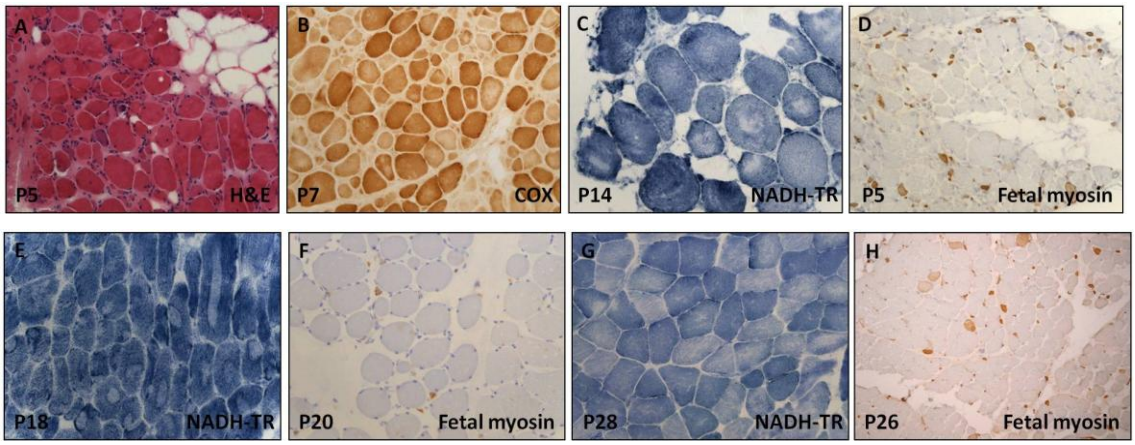


Figure 18: Muscle histopathology findings of collagen VI-related myopathies compared to *RYR1*-related and *SEPN1*-related core myopathies.

(A-D) Muscle biopsies from collagen VI-related myopathy patients 5 (P5), 7 (P7) and 14 (P14) (see Table 4 above) demonstrating dystrophic-appearing findings including increased perimysial connective tissue and perimysial and endomysial fat (A), cores and minicores in a patchy distribution (B), cores and 'unevenness' in a patchy distribution (C) and very small (<5 microns), small and large fibres positive for fetal myosin (D).

(E-F) Muscle biopsies from *RYR1*-related core myopathy patients 18 (P18) and 20 (P20) (see Table 4 above) demonstrating widespread cores (E) and very small (<5 microns) fibres positive for fetal myosin (F).

(G-H) Muscle biopsies from *SEPN1*-related core myopathy patients 28 (P28) and 26 (P26) (see Table 4 above) demonstrating widespread minicores (G) and very small (<5 microns), small and large fibres positive for fetal myosin (H).

COX = cytochrome oxidase
NADH-TR = nicotinamide adenine dinucleotide-tetrazolium reductase

(Images courtesy of Dr Rahul Phadke, Dr Lucy Feng and Mr Darren Chambers, Dubowitz Neuromuscular Centre.)

5.1.3 Discussion

This study of histological findings in collagen VI-related myopathy patients demonstrates that cores and/or minicores are common muscle histopathology findings in this patient population. Given this information, it is essential to include collagen VI-related myopathies in the differential diagnosis for patients presenting with a clinical picture of congenital weakness and cores and/or minicores on muscle biopsy.

The clinical phenotypes of infants with collagen VI-related myopathy and *RYR1*-related core myopathy can have significant overlap.[51] While muscle imaging findings can reveal different patterns of muscle involvement in these conditions, not all neuromuscular centres have expertise in muscle ultrasound, and young children are not good candidates for muscle MRI given the requirement of sedation to obtain such imaging. In the absence of muscle imaging patterns, muscle histopathology findings can offer clues for distinguishing between collagen VI-related myopathy and *RYR1*-related core myopathy. In particular, patients with collagen VI-related myopathy typically have fibres of varying sizes positive for fetal myosin: small and large; or very small (<5 microns), small and large. Patients with *RYR1*-related core myopathy classically have only very small (<5 microns) fibres positive for fetal myosin. It is, however, important to note that those patients with autosomal recessively inherited *RYR1*-related core myopathy have histopathology findings more closely resembling collagen VI-related myopathy patients. In particular, patient 22 in this series has autosomal recessively inherited *RYR1*-related core myopathy and has fetal myosin positive fibres of various sizes (very small, small and large), as frequently seen in the biopsies of patients with collagen VI-related myopathy.

The clinical phenotypes of *SEPN1*-related core myopathy patients do not overlap with those of collagen VI-related myopathy patients to the degree which *RYR1*-related core myopathy phenotypes do. The histopathology findings in *SEPN1*-related core myopathy and collagen VI-related myopathy overlap significantly, however. While the muscle of *SEPN1*-related core myopathy is classically defined by the presence of cores and minicores, this study reveals that the structural findings of cores and minicores are common in collagen VI deficiency as well. The role which patterns of fetal myosin positive fibres play in helping to distinguish between *RYR1*-related core myopathy and collagen VI-related myopathy is not applicable to *SEPN1*-related core myopathy, as both patients with *SEPN1*-related core myopathy and

collagen VI-related myopathy typically have muscle biopsy findings of very small (<5 microns), small and large fibres positive for fetal myosin. Consequently, distinguishing between *SEPN1*-related core myopathy and collagen VI-related myopathy relies heavily on clinical findings, as well as muscle imaging findings, which can be highly sensitive in identifying disease-specific patterns,[129] and not on muscle histopathology findings.

The fact that only one of the collagen VI-related myopathy patients included in this series has a clinical phenotype of Bethlem myopathy (P8) should be noted. We have seen core-like regions in the muscle biopsies of further patients with Bethlem myopathy (unpublished data). Given that the clinical course of BM patients is milder than that of UCMD and intermediate collagen VI-related myopathy patients, it is noteworthy that BM patients have the histopathology finding of cores in common with UCMD and intermediate patients.

Finally, electron microscopy (EM) studies of the core and minicore-like regions seen with light microscopy in the muscle biopsies of collagen VI-related myopathy patients would be helpful to further characterise these structural abnormalities. EM may reveal myofibrillar disorganisation, Z-line streaming and/or a decrease or absence of mitochondria corresponding to the cores and minicores seen with light microscopy. Some types of cores have only minimal myofibrillar disorganization evident with EM.[188]

Studies aimed at understanding the pathophysiology underlying the formation of cores in *RYR1*-related core myopathy suggest that cores are due to increased calcium leak and resultant uncoupling of the muscle's excitation-contraction mechanism.[196] Further studies in a zebrafish model of *RYR1*-related core myopathy have demonstrated that calcium dysregulation results in mitochondrial dysfunction and, in particular, the production of mitochondrially derived reactive oxygen species.[197] Studies performed in collagen VI-related myopathy patient-derived myoblast cultures and the *Col6a1*$^{-/-}$ mouse have demonstrated evidence of mitochondrial dysfunction.[30, 89-90] Taken together, the occurrence of cores in the muscle biopsies of collagen VI-related myopathy patients potentially may offer further evidence of mitochondrial dysfunction as a pathophysiologic mechanism underlying collagen VI deficiency. Furthermore, the establishment of cores as a common histopathology finding in the collagen VI-related myopathies will - in conjunction with clinical and muscle imaging findings (when available) - assist in refining the

challenging diagnostic pathway leading to a molecularly confirmed diagnosis of collagen VI-related myopathy.

5.2 THE MYOMATRIX

A mixture of non-collagenous glycoproteins and fibrous proteins comprise the muscle extracellular matrix (ECM) known as the 'myomatrix.' Together, these proteins act as a type of scaffold, providing structural integrity to the muscle fibre while also allowing 'myofascial force transmission' (transmission of contractile forces generated by the muscle to tendons and, in turn, bones).[198-200] Histologically, the sheet-like muscle ECM or myomatrix is the basement membrane surrounding each muscle fibre (endomysium), muscle fascicle (perimysium) as well as the muscle as a whole (epimysium). Embedded within the myomatrix are nerve branches, capillaries, fibroblasts, macrophages and satellite cells.[11, 192-193]

The basement membrane can be visualised by light microscopy and is composed of the basal lamina as well as an external reticular lamina (fibrillar reticular layer). Ultrastructurally, the basal lamina contains an electon-dense layer, the lamina densa, and a less opaque inner layer called the lamina rara, which have been identified by electron microscopy.[201] Between the basal lamina and the sarcolemma lie satellite cells, the resident muscle progenitor cells.[192-193, 202-203] The basal lamina is composed of non-fibrillar collagen (collagen IV), non-collagenous glycoproteins (laminins and nidogen) and proteoglycans (perlecan, decorin, biglycan). Collagen IV, as the most abundant protein of the basal lamina, and laminins form distinct self-assembly networks.[202] Covalent cross-links stabilise the collagen IV networks which are linked via nidogen to the laminin networks.[204] Together, these basal lamina proteins contain cell surface recognition sites for: (1.) binding other basal lamina components, (2.) anchoring reticular lamina components to the basal lamina and (3.) serving as ligands for transmembrane receptors such as integrins and dystroglycans. In this manner, the basal lamina proteins link the muscle fibre cytoskeleton to the reticular lamina.[202, 205-206]

The reticular lamina includes collagens (III, V, VI, XV, XVIII) and glycoproteins including fibronectin which lie within a proteoglycan-rich substance and bind to other reticular lamina proteins as well as basal lamina proteins, promoting the scaffolding of the ECM. Besides promoting muscle structural stability, there is expanding

evidence that the myomatrix plays important roles in muscle maintenance, regeneration and developmental processes including myogenesis and synaptogenesis, predominantly through signalling pathways.[202, 205, 207]

5.2.1 Collagen VI within the myomatrix

Resulting from a decrease or deficiency of the extracellular matrix protein collagen VI, the collagen VI-related myopathies are considered disorders of the muscle ECM or myomatrix. Collagen VI is expressed in the extracellular matrices of several tissues and tissue components including blood vessels, nerves, skin, tendons, cartilage, intervertebral discs, lenses and internal organs.[208-209] In blood vessels and skin, collagen VI microfibrils associate with basement membranes, where they appear to function as anchors.[35, 209] An immunoelectron microscopy study (using gold-labelled secondary antibodies to a mouse antibody which recognises the helical domain of human collagen VI[23]) revealed a significant increase in the concentration of type VI collagen surrounding the basement membrane of blood vessels, nerves and fat cells.[209] In cells without basement membranes, however, collagen VI has been found to have a pericellular distribution, such as around tendon fibroblasts.[210]

The exact mechanisms by which altered interactions of the myomatrix with muscle result in myomatrix-type symptoms, or mixed connective tissue and muscle symptoms, remain unclear. It is interesting to note that connective tissue disorders resulting from various extracellular matrix protein deficiencies such as Ehlers-Danlos syndrome (EDS) (resulting from tenascin X deficiency) and Marfan syndrome (fibrillin-1 deficiency) can be associated with muscle atrophy and weakness, which might suggest a type of inter-dependency between the myomatrix and the muscle itself for structural integrity as well as function. These proteins, like collagen VI, are expressed in the ECM of muscle as well as in tendons and joint capsules,[211-212] which may offer one explanation why patients with deficiencies in these proteins can present with both muscle weakness, as well as joint hyperlaxity and contractures.[146]

Neuromuscular symptoms are not only seen in EDS subtypes which result from primary deficiencies of extracellular matrix proteins, however. *FKBP14,* a recently described gene which causes a form of EDS associated with sensorineural hearing loss, codes for an endoplasmic reticulum (ER) protein. *In vitro* studies performed in

skin fibroblasts demonstrate that a deficiency of FKBP14 disrupts the composition of the extracellular matrix proteins including collagen VI.[213] In particular, the clinical phenotypes of patients with mutations in *FKBP14* share similarities with UCMD patients including follicular hyperkeratosis, hypertrophic and atrophic scarring, joint hypermobility, kyphoscoliosis, congenital hypotonia, weakness and delayed motor development. Muscle biopsies from patients with this EDS subtype demonstrate findings which range from variation in fibre size and areas of decreased oxidative staining (core-like areas) to fibre atrophy and increased fat. The authors of this study hypothesise that FKBP14's role in protein folding in the ER may affect the assembly of the extracellular matrix of skin, joints, muscles, vessels, ears and eyes and, in this way, underlie the clinical manifestations of this EDS subtype.[213]

The collagen VI myopathies are indeed a clinical hybrid of connective tissue and muscle conditions. Given the expression of collagen VI in the extracellular matrix of muscle, tendons and cartilage and skin, it seems plausible that a primary deficiency of collagen VI may alone account for the muscle, joint and skin manifestations seen in patients with collagen VI-related myopathies. What is more difficult to explain, however, is how even in patients with completely absent collagen VI expression, vascular pathology has not been encountered, as collagen VI is also expressed in the extracellular matrix of vessels. This finding suggests that the absence or aberrant formation of collagen VI does not necessarily result in pathology in all tissues where collagen VI is expressed and points toward an apparent tissue-specific function of collagen VI. This theory would not only help to explain the absence of vascular pathology, but also might help to explain the lack of cardiac and nerve involvement in collagen VI-related myopathies despite the recognised expression of collagen VI in the heart[214] and nerves.[209]

5.2.2 Collagen VI mouse model studies of tendons and cartilage

The collagen VI-related myopathy mouse model (*Col6a1*$^{-/-}$) has demonstrated evidence of abnormal tendon morphology, namely disrupted cellular organisation and fibril formation. Biomechanical analyses of *Col6a1*$^{-/-}$ tendons have revealed a reduction in maximum load as well as stiffness when compared to wild type tendons.[87] Further *Col6a1*$^{-/-}$ mouse studies have revealed evidence of osteoarthritis developing at an accelerate rate, suggesting that collagen VI plays an important role in the mechanical stabilisation of the pericellular matrix of articular cartilage.[215] The

extent to which these findings may account for the hypermobility or hyperlaxity of the distal joints and proximal joint contractures seen in humans with collagen VI deficiency is unknown.

5.2.3 Integrin α7β1: a myomatrix neighbour

Integrin α7β1 is known to play important roles in anchoring the intracellular cytoskeleton to the extracellular matrix as well as in signalling cascades. Integrins are important transmembrane glycoproteins of heterotrimeric structure, containing non-covalently associated α (120-170 kDa) and β (90-100 kDa) subunits.[11, 205, 216] Integrin α7β1 is present in both skeletal and cardiac muscle and is known to bind laminin.[217] Intracellularly, integrin α7β1 binds to cytoskeletal proteins including vinculin, talin and α-actinin.[204] Extracellularly, it binds to laminin α2 and may interact with collagen IV and fibronectin, which in turn binds to tenascin X.

ITGA7 codes for α7 integrin, which through developmentally regulated RNA splicing results in different isoforms (3 cytoplasmic domains: α7A, α7B and α7C; 2 alternative extracellular domains: α7X1 and α7X2).[218-221] The α7A and α7B isoforms are reportedly concentrated at myotendinous junctions[222] and also are present at the sarcolemmal membrane and in neuromuscular junctions.[220] In particular, the α7B integrin isoform is expressed in skeletal and cardiac muscle as well as in the vasculature and in the central nervous system.[223-225] The β1D integrin isoform is specifically expressed in skeletal and cardiac muscle where it associates with the α7 subunit and is found at the costameres as well as the myotendinous and neuromuscular junctions and in cardiac muscle where it localises to costameres and intercalated discs.[226-229] The expression of α7B is developmentally regulated in myocytes and reportedly not fully expressed in skeletal muscle until 2 years of age.[188, 230]

Studies have suggested complementary structural and functional roles of dystrophin and integrin α7B, particularly given the upregulation of integrin α7B in the dystrophin-deficient skeletal muscle of patients with Duchenne muscular dystrophy (DMD) and *mdx* mice.[231] Interestingly, increased expression of integrin α7B was even noticed in DMD patients less than 2 years of age.[230] Dystrophin, like integrin α7B, mediates the connection of the muscle cytoskeleton with the extracellular matrix, although from a position along the cytoplasmic side of the muscle plasma membrane. This linkage

occurs via the dystrophin glycoprotein complex (DGC), a complex of glycoproteins including dystroglycans, sarcoglycans and syntrophins.[232]

Conversely, studies of laminin α2 deficiency performed in both human and murine muscle (*dy*$^{-/-}$ and *dy*$^{2J-/-}$ mouse models) have demonstrated a secondary decrease in integrin α7β1 expression.[221, 230, 233] Furthermore, a study of transgenic overexpression of integrin α7 in the *dy*$^{W-/-}$ mouse model of merosin-deficient CMD revealed a reduction in muscle pathology, maintenance of muscle strength and function and an improvement of life expectancy.[234]

In a study of 117 patients with 'unclassified' congenital myopathy and congenital muscular dystrophy, 3 patients (ages 4 years, 11 years and 8 months, respectively) were found to have evidence of integrin α7 deficiency (using antibodies against α7A, α7B and β1D) and normal laminin α2 expression.[235] These patients were originally described with phenotypes consistent with congenital myopathies; however, a follow-up paper reported that the subsequent severe clinical course of one of the patients was more consistent with a congenital muscular dystrophy than a congenital myopathy.[236] Mice lacking integrin α7 demonstrate a progressive muscular dystrophy as well as a disruption of myotendinous junctions.[237]

In a further, large study evaluating 210 muscle biopsies obtained from patients with muscular dystrophies and myopathies of unknown aetiology, integrin α7B expression was decreased in 35 patients (17%) and absent in 6 patients (3%).[238] Interestingly, all 6 patients with absent integrin α7B expression were muscular dystrophy patients (based on muscle biopsy evidence of muscle fibre degeneration and regeneration) with 4 of the patients more than 2 years of age at the time of muscle biopsy and 2 of the patients less than 2 years of age at the time of biopsy. Sequencing for integrin α7B mutations revealed only one *ITGA7* missense mutation with no further mutation identified on the second allele. Based on these results, this study concluded that a secondary integrin α7 deficiency is common in muscular dystrophies and myopathies of unknown aetiology.[238]

A further study evaluated integrin α7 expression in a cohort of unsolved congenital muscular dystrophy patients and found that 31/45 patients (69%) had altered expression of integrin α7. Twelve patients (27%) had completely absent integrin α7 expression; however, none of these patients were found to have mutations in *ITGA7.*[8] This is similar to our experience in a cohort of unsolved congenital

myopathy patients studied at our neuromuscular centre. In particular, 14/58 (24%) congenital myopathy patients of unknown genetic aetiology (whose clinical phenotypes I studied in detail and who were over 2 years of age at the time of biopsy) had absent sarcolemmal integrin α7B expression (unpublished results; Dr Francesco Conti).

5.2.4 Hypothesis on the effect of collagen VI deficiency on integrin α7 expression

While integrin α7β1 is not known to bind to collagen VI, other members of the integrin family, including integrins α1β1 and α2β1, may mediate cell binding to collagen VI, and integrins α5β1 and αvβ3 bind to collagen VI via a hidden Arg-Gly-Asp motif.[239-240] Given that integrin α7β1 is an important transmembrane protein which links the extracellular matrix and the intracellular cytoskeleton, I hypothesised that the myopathic findings evident on histological studies of collagen VI-related myopathy patients could be exacerbated by a generalised destabilisation of the myomatrix resulting from collagen VI deficiency. While studies of Duchenne muscular dystrophy (both in patient muscle and muscle from the *mdx* mouse) have reported an increased expression of integrin α7B, studies of models of merosin-deficient CMD have revealed a decreased expression of integrin α7B in the absence of the myomatrix protein laminin-α2. Since collagen VI, like laminin-α2 is an extracellular matrix protein, I hypothesised that integrin α7B would similarly be decreased in the setting of collagen VI deficiency. Furthermore, I hypothesised that a concomitant decrease in the transmembrane protein integrin α7B may result in further destabilisation of the myomatrix, and thereby contribute to the muscle pathology seen in patients with collagen VI deficiency.

To investigate this hypothesis, I (in collaboration with Dr Francesco Conti, see Collaborators, page 5) performed immunohistochemical studies looking at integrin α7B expression in muscle biopsies from patients with confirmed mutations in *COL6A2*. Integrin α7B expression in collagen VI deficient muscle has not been reported in the literature. Furthermore, considering the possible therapeutic implications of potentially decreased integrin α7 in collagen VI-related myopathies, given the findings of transgenic overexpression of integrin α7 in a merosin-deficient

CMD mouse model,[234] this hypothesis seemed to be an important research question to study.

5.2.5 Materials and Methods

I obtained clinical information from two patients with autosomal recessively inherited UCMD followed in our neuromuscular centre (the Dubowitz Neuromuscular Centre) as part of an ethically approved study. Sanger sequencing of the collagen VI genes was performed in a diagnostic laboratory (Guy's and St Thomas' DNA Laboratory, London) on genomic DNA extracted from blood taken from the patients and their unaffected parents using single condition amplification/internal primer (SCAIP) sequencing as previously reported.[74] Consent was obtained for performing diagnostic as well as research studies on the muscle biopsy sample for both patients.

Muscle biopsy sections were stained and immunolabelled as described.[241] Collagen VI immunohistochemical studies were performed using monoclonal antibodies to collagen VI, and double-labelling with perlecan was performed as described.[118] Further immunohistochemical staining was performed using collagen VI α1, α2 and α3 chain-specific antibodies (in collaboration with Dr L Feng and Mr D Chambers; see Collaborators, page 5) and integrin α7B antibodies (in collaboration with Dr F Conti, see Collaborators, page 5). The properties of the antibodies used are listed below (Table 5). Muscle sections were viewed with an epifluorescence microscope: Leica Digital Module R microscope with MetaMorph software (Universal Imaging).

Antibody	Clonality	Source	Epitope(s)	Company
collagen VI	monoclonal	mouse	not mapped	Millipore (MAB3303)
collagen VI	monoclonal	mouse	not mapped	Millipore (MAB1944)
collagen VI α1	polyclonal	rabbit	not mapped	Lifespan Biosciences (LS-B696)
collagen VI α2	monoclonal	mouse	not mapped	Abnova (H00001292-M01)
collagen VI α3	polyclonal	rabbit	not mapped	Sigma-Aldrich (HPA010080)
perlecan (anti-heparan sulfate proteoglycan)	monoclonal	rat	perlecan domain 4	Merck Millipore (MAB1948)
integrin α7B	polyclonal	rabbit	(intracellular domain)	(gift from Dr Ulrike Mayer)

Table 5: Properties of Antibodies.

5.2.6 Results

An infant girl (patient 30) with a phenotype of UCMD was found to have autosomal recessive loss-of-function mutations in *COL6A2* [c.1170+1delG in intron 23 and c.2386A>T (p.Lys796X) in exon 26], which parental testing confirmed were present in trans. A boy (patient 31) with a phenotype of UCMD was found to also have autosomal recessively *COL6A2* mutations (homozygous mutation: r.2839_2850del; p.Leu947Gly950del). (See Table 6 for details of clinical phenotypes.)

Patient 30 had a large muscle biopsy performed at 6 months of age at an outside hospital which was sent to our neuromuscular centre for further analyses. Haematoxylin and eosin (H&E) staining of this muscle biopsy revealed the presence of an intramuscular nerve as well as an artery and a vein (Figure 19). There was evidence of variation in fibre size and excess connective tissue. Oxidative stains revealed evidence of core-like areas, some fibres with central accumulation of mitochondria as well as fibres with a lobulated-like appearance. There were fetal myosin positive fibres varying in size from very tiny fibres (<5 microns) to large fibres. Immunohistochemical double-labelling with collagen VI and perlecan antibodies revealed a complete absence of collagen VI expression at the sarcolemma. While there was no evidence of collagen VI expression (with either antibody) on the intramuscular nerve, the vessels present in the muscle biopsy demonstrated striking collagen VI expression (Figure 20), with particularly strong expression along an intramuscular artery (Figure 21).

Patient 31 underwent a muscle biopsy at our neuromuscular centre at 2 years of age. H&E staining revealed variation in fibre size and occasional split fibres. There was evidence of excess fat and connective tissue. Oxidative stains revealed core-like regions. While immunohistochemical double-labelling with collagen VI and perlecan demonstrated decreased collagen VI expression at the sarcolemma, collagen VI expression was not entirely absent (not shown).

Patient	Sex	Current age (years)	Collagen VI mutations	Motor Function	Contractures	Hyperlaxity	Skin Findings
30	Female	1.6	*COL6A2* [c.1170+1delG in intron 23 and c.2386A>T (p.Lys796x) in exon 26]	antigravity limb strength; unable to sit without support	elbows and knees	significant at distal joints	none appreciated
31	Male	10.6	*COL6A2* (homozygous mutation: r.2839_2850del; p.Leu947Gly950del)	sat at 10 months; crawled at 22 months; walked with gaiters at 3 years; took first independent steps at 5 years; presently wheelchair dependent	elbows and knees	significant at distal joints	follicular hyperkeratosis

Table 6: Patient clinical phenotypes and corresponding *COL6A2* genotypes for two autosomal recessive Ullrich CMD patients.

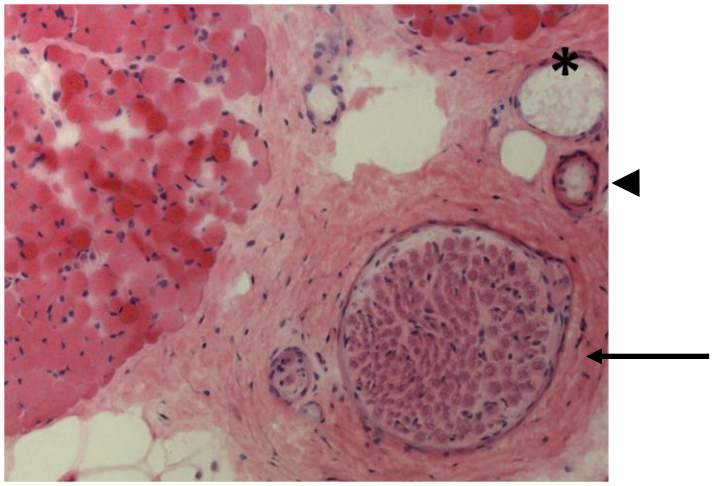


Figure 19: H&E staining of muscle of patient 30.

There is evidence of an intramuscular nerve (arrow), artery (arrowhead) and vein (asterisk).

(Courtesy of Dr Rahul Phadke and Mr Darren Chambers, Dubowitz Neuromuscular Centre.)

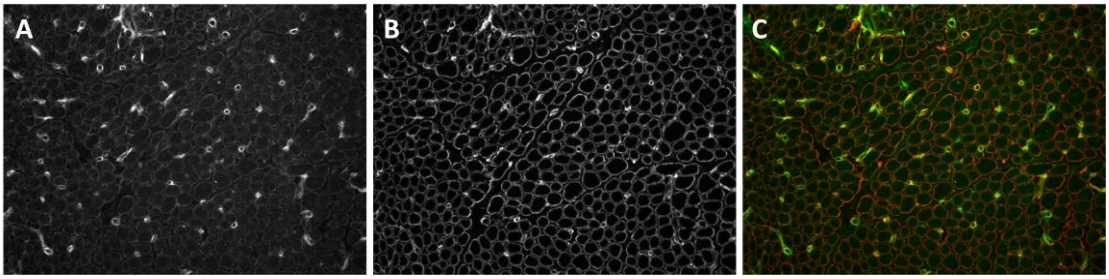


Figure 20: Collagen VI Immunohistochemical studies of the muscle biopsy of patient 30.

Staining with collagen VI antibody (MAB 1944) (A), perlecan antibody (B) and double-labelling with collagen VI (green) and perlecan (red) antibodies (C), demonstrating an absence of collagen VI expression along the sarcolemma with retention of collagen VI expression on intramuscular vessels.

(Courtesy of Dr Rahul Phadke and Mr Darren Chambers, Dubowitz Neuromuscular Centre.)

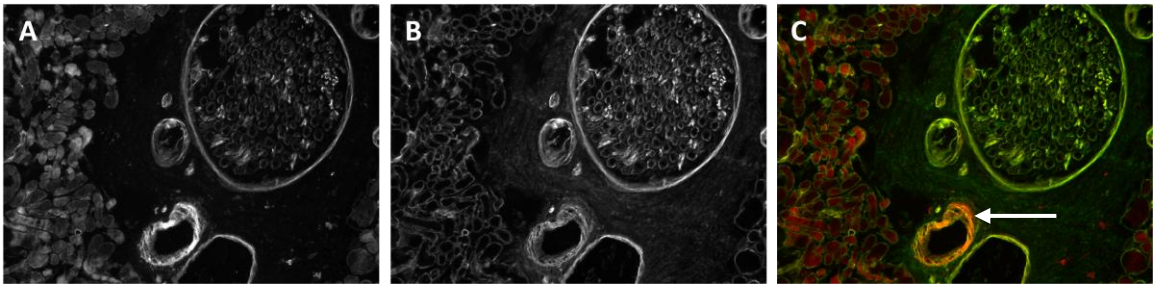


Figure 21: Collagen VI immunohistochemical studies of a muscle biopsy sample from patient 30 containing an intramuscular nerve, artery and vein.

Staining with collagen VI antibody (MAB 3303) (A), perlecan antibody (B) and double-labelling with collagen VI (red) and perlecan (green) antibodies (C), demonstrating an absence of collagen VI expression along the sarcolemma and along the large intramuscular nerve with retention of collagen VI expression on an intramuscular artery (arrow).

(Courtesy of Dr Rahul Phadke and Mr Darren Chambers, Dubowitz Neuromuscular Centre.)

Further immunohistochemical staining with collagen VI chain-specific antibodies was undertaken (Figure 22) to elucidate whether the retained expression of collagen VI was due to α1 and/or α3 chains. Given patient 30's compound heterozygous mutations in *COL6A2* (a missense mutation predicted to result in aberrant splicing in combination with a stop mutation on the other allele), one would expect that the collagen α2 chain would not be formed. Furthermore, given the reliance of the α1 and α3 chains on the presence of the α2 chain to form a heterotrimeric monomer in the initial step of the complex assembly process of the collagen VI protein, the absence of one of the three collagen VI chains would be predicted to result in no production of the collagen VI tetramer, termed a 'functional null.'

One potential explanation for the retained expression of collagen VI along the blood vessels in patient 30 could be that of tissue-specific collagen VI expression resulting from *COL6A2* splice variants. As stated in Chapter 1 (section 1.4.2), alternative splicing of *COL6A2* results in 3 recognised collagen VI α2 chains: α2C2, α2C2a and α2C2a'.[84] Our present understanding of alternative splicing in *COL6A2* indicates that the 'variable' region (where alternative splicing occurs) begins in exon 28.[84] Patient 30's deletion in intron 23 of *COL6A2* is predicted to cause aberrant splicing while her exon 26 mutation causes a premature stop. Given the location of these mutations upstream from exon 28, the known *COL6A2* splice variants (α2C2, α2C2a and α2C2a') would not be responsible for the apparent collagen VI expression seen along intramuscular blood vessels in patient 30.

In theory, however, the formation of a *COL6A2* splice variant (by a splice variant beyond those variants recognised to date) might explain how residual collagen VI α2 expression could occur. Furthermore, the mRNA variants resulting from alternative splicing demonstrate variable expression between different tissue types[242-244] which may explain how collagen VI expression is present only on the intramuscular vessels and not on the sarcolemma or the intramuscular nerves. Some authors have hypothesised that alternative splicing of *COL6A2* may result in different functional properties of collagen VI α2.[245] Alternative spliced *COL6A2* hypothetically could bind with collagen VI α1 and α3 chains, enabling the formation of the collagen VI tetramer.

Interestingly, staining with an α1 chain-specific antibody revealed subtle residual expression on intramuscular vessels only (with no expression along the sarcolemma). No expression of the α2 chain or the α3 chain was appreciated either along the sarcolemma or on intramuscular vessels (Figure 23). The apparent

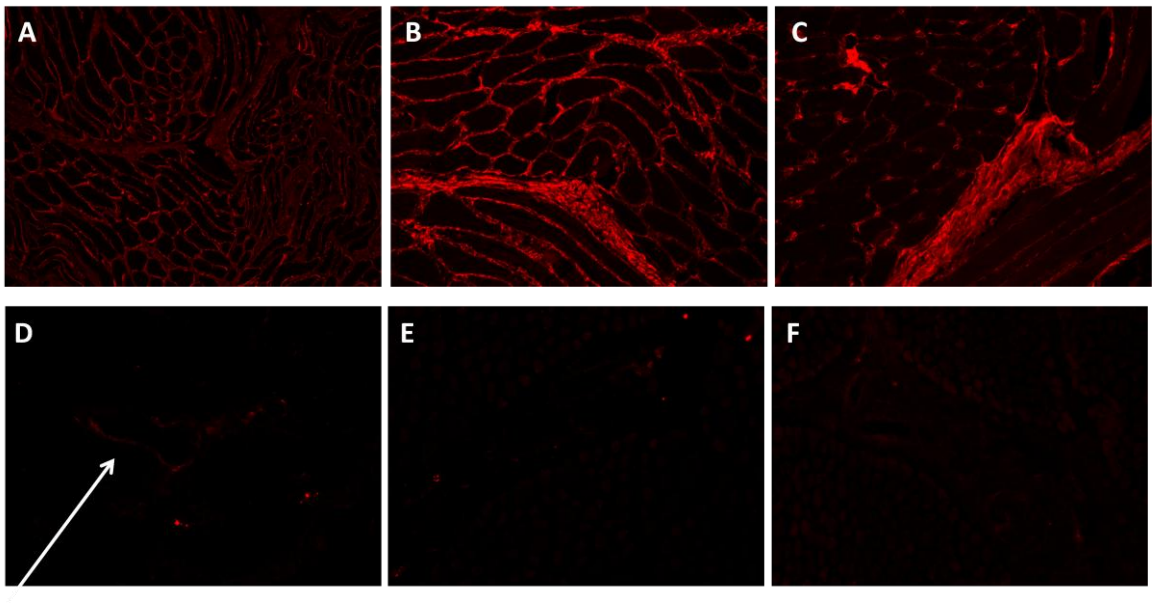


Figure 22: Immunohistochemical studies with collagen VI chain-specific antibodies.

Staining of control muscle with antibodies for collagen VI α1 (A), α2 (B) and α3 (C). Staining of muscle from patient 30 with antibodies for collagen VI α1 (D), α2 (E) and α3 (F). There is evidence of some expression of the collagen α1 chain along an intramuscular vessel of patient 30 (D - arrow).

(Courtesy of Dr Rahul Phadke and Mr Darren Chambers, Dubowitz Neuromuscular Centre.)

expression of the collagen VI α1 antibody along the vessel wall (Figure 23) is challenging to explain. Cross-reactivity of the of the collagen VI antibody to another protein expressed along the vessel wall is one possibility, which seems probable given that the collagen VI α1 antibody used is a polyclonal antibody, and the epitopes of this antibody are not mapped (Table 5).

Staining with an integrin α7B antibody (Table 5) was performed on muscle from UCMD patients 30 and 31 and compared to staining in control muscle (from a teenage individual without neuromuscular disease). Integrin α7B expression was notably increased in patient 30 and in patient 31 (Figure 23). This result contradicted my hypothesis of secondarily decreased integrin α7 expression in collagen VI deficiency. Furthermore, it is important to note that patient 30's muscle biopsy was performed at 6 months of age, which is an age when the developmentally regulated expression of integrin α7B is not typically evident (see section 5.2.3).[188, 230] Patient 31 was 2 years at the time of his muscle biopsy, and integrin α7B expression is also notably increased in his muscle biopsy (Figure 23).

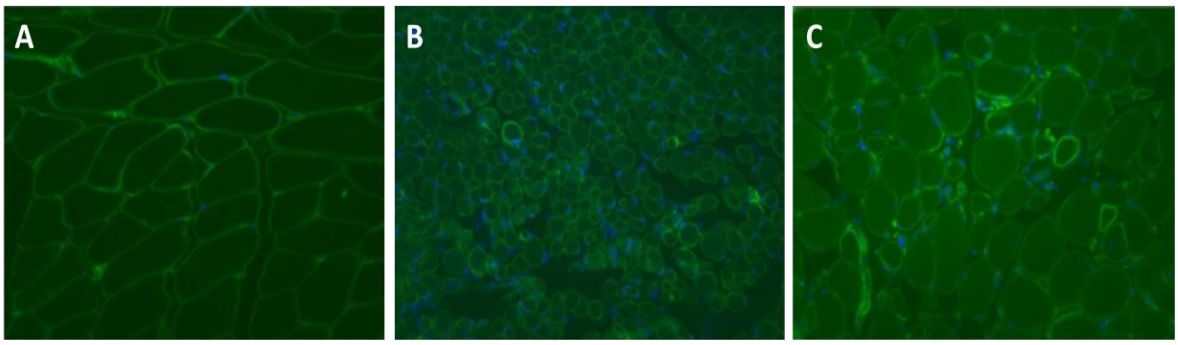


Figure 23: Expression of integrin α7B in patients with autosomal recessively inherited Ullrich congenital muscular dystrophy.

Control muscle (from a teenage individual without neuromuscular disease) reveals normal integrin α7B expression (A). Muscle from UCMD patient 30 at 6 months of age demonstrates apparent upregulation of integrin α7B expression somewhat uniformly (B) while muscle from UCMD patient 31 at 2 years of age demonstrates apparent patchy upregulation of integrin α7B expression.

(Courtesy of Dr Francesco Conti, Dubowitz Neuromuscular Centre.)

5.2.7 Discussion

In UCMD patient 30 a complete absence of collagen expression would be expected, given her compound heterozygous loss-of-function mutations (namely a deletion resulting in aberrant splicing and a stop mutation) which result in a state of 'functional null' for the collagen VI protein. The apparent retention of collagen VI expression, however, on patient 30's intramuscular vessels in the setting of absent sarcolemmal expression (using monoclonal antibodies purported to bind to the collagen VI tetramer) is striking. One potential explanation is that of *COL6A2* splice variants which could have tissue-specific expression. Given that the epitope for these collagen VI antibodies have not been mapped (Table 5), there remains a possibility that these antibodies are cross-reacting with vascular proteins.

It is important to note, however, that apparently retained expression of collagen VI on intramuscular vessels in the setting of absent sarcolemmal expression is not unprecedented, as this finding has been described in two UCMD patients with absent sarcolemmal expression of collagen VI using a monoclonal antibody for the triple helical domain of collagen VI (Fuji Chemical, Japan).[189] Interestingly, one of UCMD patients reported with these similar immunohistochemical findings harboured a homozygous *COL6A2* mutation (26 base pair deletion in exon 14, resulting in a frameshift and a premature stop codon).[246]

We undertook further studies, using collagen VI chain-specific antibodies and found evidence of subtle expression of the collagen VI α1 chain on an intramuscular vessel (Figure 22). Again, antibody cross-reactivity remains possible especially in light of the fact that the collagen VI α1 antibody is polyclonal. The use of other chain-specific antibodies is necessary for determining significance of this finding. In particular, given the strong expression of collagen VI α1, α2 and α3 on the control muscle, the expression of the collagen VI α1 antibody appears subtle and certainly not as strong as the apparent expression of collagen VI on the intramuscular vessels with collagen VI antibodies (Table 5), which likely bind to collagen VI tetramers, although the epitopes of the two monoclonal collagen VI antibodies used are not mapped.

As these results are based on the assessment of a limited number of cases, further studies are necessary using different collagen VI antibodies in order to evaluate

whether or not this result is simply reflective of antibody cross-reactivity. To date, tissue-specific expression of collagen VI has not been reported; however, detailed studies evaluating collagen VI expression in muscle, nerve, skin and tendons in patients with collagen VI deficiency have not been performed. Furthermore, even in the absence of known tissue-specific expression in wildtype collagen VI α2 transcripts, tissue-specific expression of collagen VI α2 splice variants are predicted to occur.[245] Patients with complete absence of sarcolemmal collagen VI expression, or collagen VI 'functional nulls' afford an opportunity to further investigate the question of tissue-specific collagen VI expression.

As stated above (section 5.1.1), it is intriguing that patients with collagen VI deficiency have not been reported to manifest vascular pathology given that collagen VI is expressed in the extracellular matrix of blood vessels. Instead, the expression of collagen VI in muscle, tendons and skin appears to play a definitive role in the phenotypic characteristics manifested in patients with collagen VI deficiency. In particular, a tendency towards vascular fragility and bleeding has not been noted in collagen VI-related myopathy patients nor reported in the collagen VI mouse model (*Col6a1*$^{-/-}$).[86] In a cohort of 57 molecularly confirmed collagen VI patients, which I studied in the United States, only one patient had a history significant for vascular pathology. That particular patient had a clinical phenotype of UCMD and unfortunately suffered a fatal cerebral aneurysmal bleed at the age of 40 years (unpublished data). As cerebral aneurysms can be inherited independently, it is not possible to determine from one case whether or not there is an association between collagen VI deficiency and vascular malformations.

It remains possible that if a proclivity towards vascular events does exist in collagen VI-related myopathy patients, such vascular vulnerability might be a function of patient age and/or the severity of the collagen VI deficiency. Due to the fact that the UCMD patient population I studied in the United States was skewed toward young ages, with all but 2 patients below 25 years of age, an accurate study of prevalence of associated vascular pathology could not be performed. While screening for the presence of cerebral vascular malformations in our collagen VI-related myopathy cohort was considered, the complicated issues surrounding magnetic resonance imaging in this patient population (including the need for non-invasive ventilation to lie recumbent for the duration of an MRI scan and the high likelihood of metallic spinal instrumentation) precluded this screening from being carried out. With this in mind, and given the lack of data surrounding the role of collagen VI in the

extracellular matrix of vessels, *in vivo* studies of collagen VI vascular expression would be valuable and could be performed in muscle biopsy samples from collagen VI related myopathy patients.

Studies of integrin α7B expression in human muscle have revealed an absence of integrin α7B expression in the sarcolemma until 2 years of age but strong staining of integrin α7B in vessels (medium calibre/non-capillary) in fetal skeletal muscle.[230] Furthermore, whilst integrin α7β expression was found to be reduced along the sarcolemma of patients with laminin α2 deficiency, integrin α7B expression was evident on intramuscular vessels.[230] Integrin α7β expression on intramuscular vessels appears to be present prior to the sarcolemmal expression of integrin α7B and sustained in the absence of sarcolemmal laminin α2, suggesting both developmental regulation and tissue-specific expression. While collagen VI is not known to be developmentally regulated, the question of tissue-specific expression has not been fully explored.

The increased expression of integrin α7B in collagen VI-deficiency is a finding which offers evidence, although preliminary, towards disproving my hypothesis of secondarily decreased expression of integrin α7B in collagen VI-related myopathies. While both collagen VI and laminin α2 are myomatrix proteins, it is important note that integrin α7B is a receptor of laminin α2 [247] but is not known to be a receptor for collagen VI. It is also important to point out that the binding between integrin α7B and laminin α2 is functional as well as mechanical, which is highlighted by the fact that the degree of integrin α7B reduction observed in the muscle of laminin α2 deficient CMD patients was not correlated with the degree of laminin α2 expression.[230]

Clearly, these results are only preliminary, and studies of integrin α7B expression must be performed in a larger sample of muscle obtained from collagen VI-related myopathy patients, with collagen VI deficiency resulting from different mutational mechanisms and mutations occurring in the three different collagen VI chains [α1(VI), α2(VI) and α3(VI)]. If, indeed, a more extensive study of integrin α7B expression in collagen VI deficiency replicates these results, then transgenic overexpression of integrin α7B would not be considered a viable therapeutic strategy for the collagen VI-related myopathies. Various strategies aiming at stabilising the transmembrane bridge which links the muscle cytoskeleton to the extracellular matrix have been undertaken.[204] In particular, the transgenic overexpression of integrin α7 in the $dy^{W-/-}$

mouse model for laminin α2 deficiency has demonstrated promising results.[234] Whilst these preliminary results of integrin α7B upregulation in collagen VI deficient muscle raise uncertainty about the potential therapeutic role of integrin α7β in collagen VI deficiency, the potential role of upregulation of other myomatrix proteins in stabilising the cytoskeleton-extracellular matrix link should be considered.

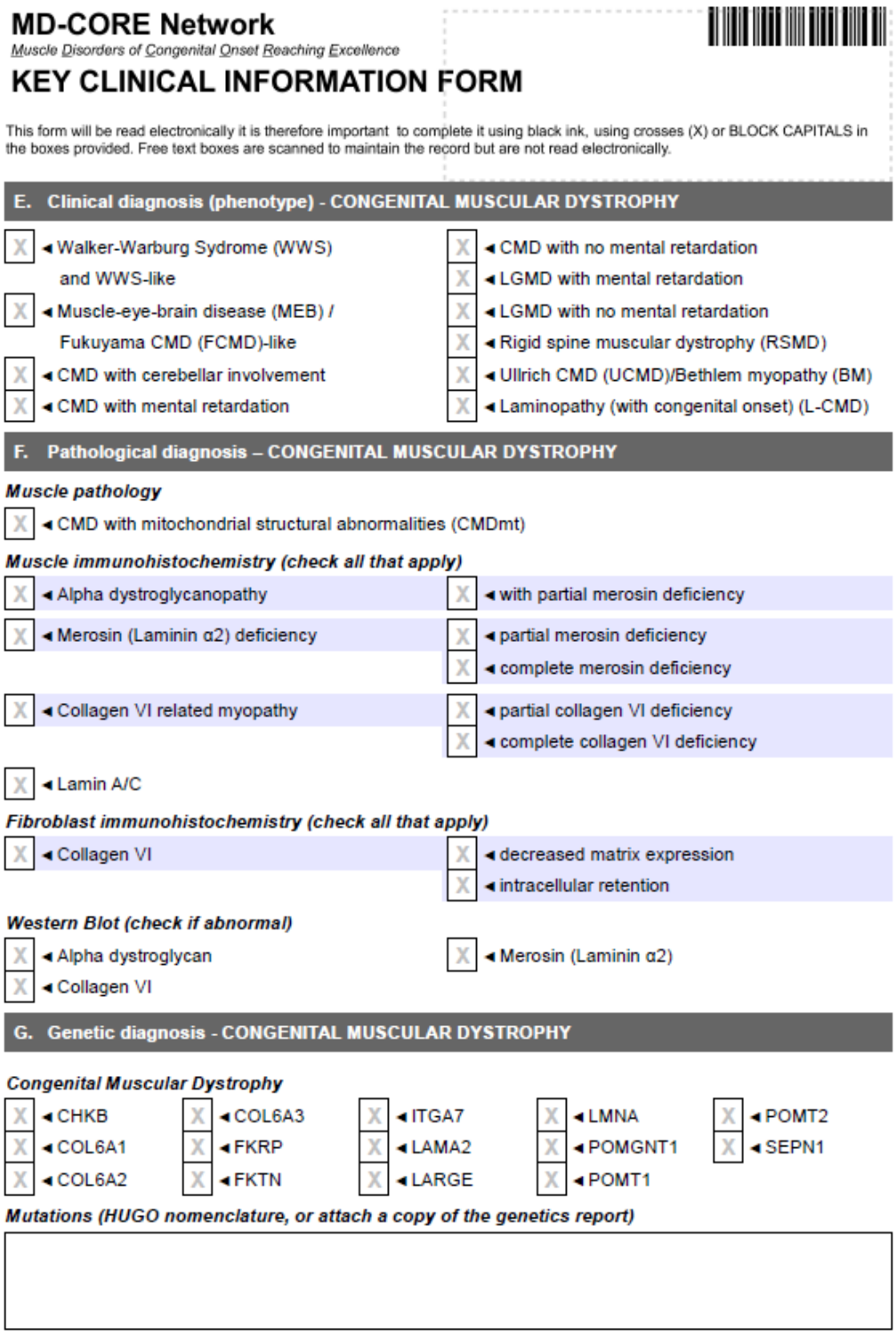
Decorin and biglycan are leucine-rich proteoglycans of the myomatrix which bind close to the N terminus of the collagen VI triple helical region.[33] Furthermore, biglycan has been found to promote the organisation of collagen VI into a hexagonal-like network.[248] Since biglycan binds to collagen VI and also interacts with the dystroglycan complex as well as the sarcoglycan complex, collagen VI may be indirectly linked- via biglycan and the dystrophin associated protein complex- to the sarcolemma receptors.[249-250] Given these reported interactions, the upregulation of biglycan might hold therapeutic promise in the collagen VI-related myopathies as a means of stabilising the essential cytoskeleton-myomatrix link. Furthermore, given the presence of biglycan in the extracellular matrices of cartilage, tendons, bones and teeth as well as muscle,[251] perhaps the upregulation of biglycan might also have beneficial effects on the tendon and cartilage pathologies seen in collagen VI deficiency. Studies of recombinant non-glycanated biglycan[251] in the collagen VI-related myopathy *Col6a1*$^{-/-}$ mouse model may begin to address these questions.

CHAPTER 6: CONCLUSION

6.1 NAVIGATING THROUGH CLINICAL MANIFESTATIONS

While the collagen VI-related myopathies are among the most common of the congenital muscular dystrophy subtypes[4-6] (with the highest relative frequency among CMD subtypes in the UK),[9] they are indeed 'rare' diseases (defined by the European Commission on Public Health as having a prevalence of less than 5 affected persons per 10,000 individuals in a community[252] and defined by the US Congress by less than 200,000 affected persons in the US[253]). The journey towards clinical trials for rare diseases begins with the recognition of the condition's clinical phenotype and is followed by efforts to arrive at a genetic confirmation of the diagnosis. Extensive natural history data is the subsequent challenge facing rare diseases, and it is an essential hurdle to overcome in order to progress toward clinical trials.

In the UK, a network of neuromuscular centres has been established for coordinating natural history data collection in neuromuscular conditions. Through the efforts of this 'UK Neuromuscular Network' and support from the Muscular Dystrophy Campaign and the Medical Research Council (MRC) Centre for Neuromuscular Diseases, tools for gathering prospective natural history data in Duchenne muscular dystrophy patients and spinal muscular atrophy patients have been established and have already provided essential information for defining motor function natural history[254-255] and optimising clinical care. In order to extend natural history data collection to the collagen VI-related myopathies as well as all congenital muscular dystrophies and all congenital myopathies, I created data entry forms for gathering prospective natural history data in these patient populations. A 'Key Clinical Information Form' is used for entering the clinical history and diagnostic testing results at the time of the patient's initial assessment (Figure 24) while the 'Medical Information Form' is used for entering clinical information at subsequent clinical evaluations (Figure 25). I entitled this initiative 'MD-CORE: Muscle Disorders of Congenital Onset Reaching Excellence' because the natural history data gathered through the use of these forms increases the potential of neuromuscular centres of reaching excellence of clinical care and clinical research. With the assistance of a computer database design company (Certus Technology) these data entry forms have been converted into 'scannable' forms, allowing the data to be scanned into a computer and stored in a secure database, thus greatly facilitating the data collection process.



MD-CORE Network

Muscle Disorders of Congenital Onset Reaching Excellence

KEY CLINICAL INFORMATION FORM

This form will be read electronically it is therefore important to complete it using black ink, using crosses (X) or BLOCK CAPITALS in the boxes provided. Free text boxes are scanned to maintain the record but are not read electronically.

E. Clinical diagnosis (phenotype) - CONGENITAL MUSCULAR DYSTROPHY

- X ◂ Walker-Warburg Sydrome (WWS) and WWS-like
- X ◂ Muscle-eye-brain disease (MEB) / Fukuyama CMD (FCMD)-like
- X ◂ CMD with cerebellar involvement
- X ◂ CMD with mental retardation
- X ◂ CMD with no mental retardation
- X ◂ LGMD with mental retardation
- X ◂ LGMD with no mental retardation
- X ◂ Rigid spine muscular dystrophy (RSMD)
- X ◂ Ullrich CMD (UCMD)/Bethlem myopathy (BM)
- X ◂ Laminopathy (with congenital onset) (L-CMD)

F. Pathological diagnosis – CONGENITAL MUSCULAR DYSTROPHY

Muscle pathology

- X ◂ CMD with mitochondrial structural abnormalities (CMDmt)

Muscle immunohistochemistry (check all that apply)

- X ◂ Alpha dystroglycanopathy
  - X ◂ with partial merosin deficiency
- X ◂ Merosin (Laminin α2) deficiency
  - X ◂ partial merosin deficiency
  - X ◂ complete merosin deficiency
- X ◂ Collagen VI related myopathy
  - X ◂ partial collagen VI deficiency
  - X ◂ complete collagen VI deficiency
- X ◂ Lamin A/C

Fibroblast immunohistochemistry (check all that apply)

- X ◂ Collagen VI
  - X ◂ decreased matrix expression
  - X ◂ intracellular retention

Western Blot (check if abnormal)

- X ◂ Alpha dystroglycan
- X ◂ Collagen VI
- X ◂ Merosin (Laminin α2)

G. Genetic diagnosis - CONGENITAL MUSCULAR DYSTROPHY

Congenital Muscular Dystrophy

X ◂ CHKB	X ◂ COL6A3	X ◂ ITGA7	X ◂ LMNA	X ◂ POMT2
X ◂ COL6A1	X ◂ FKRP	X ◂ LAMA2	X ◂ POMGNT1	X ◂ SEPN1
X ◂ COL6A2	X ◂ FKTN	X ◂ LARGE	X ◂ POMT1	

Mutations (HUGO nomenclature, or attach a copy of the genetics report)

Figure 24: A page from the MD-CORE Key Clinical Information data entry forms.

On this form clinicians enter the diagnostic information available (clinical, pathological and/or genetic) for patients with different forms of congenital muscular dystrophies. Other pages of the Key Clinical Information Form contain areas for recording details about prenatal/birth history, signs at birth, presenting symptoms, family history, investigations and diagnostic samples collected.

MD-CORE Network

Muscle Disorders of Congenital Onset Reaching Excellence

MEDICAL INFORMATION FORM

This form will be read electronically it is therefore important to complete it using black ink, using crosses (X) or BLOCK CAPITALS in the boxes provided. Free text boxes are scanned to maintain the record but are not read electronically.

Please refer to the instructions and guidance on the cover sheet of this assessment before you complete the form. Please use BLACK INK to complete this form.

A. Patient & assessment details

Assessed by ▼

▼Date of Assessment

D	D	M	M	Y	Y	Y	Y

Relevant Interim History ▼

B. Motor function at present time

	No▼	*▼Yes*	*If yes, ▼*							
Able to run/jump	X	X								
Walk independently	X	X								
Walk with orthotics only (not including calipers)	X	X								
Walk with calipers (or walker) only	X	X	calipers only ►	X	walker only ►	X				
Part-time wheelchair use	X	X	from age ►	Y	Y	M	M			
Full-time wheelchair use	X	X	from age ►	Y	Y	M	M			
Sitting independently	X	X								
Sitting with support	X	X								

C. Strength

	No▼	*▼Yes*	*If yes, explain▼*
Overall stable	X	X	
Steady worsening	X	X	
Acute worsening	X	X	
Steady improvement	X	X	
Acute improvement	X	X	

Figure 25: A page from the MD-CORE Medical Information data entry forms.

On this form clinicians enter information regarding interim changes and current functioning. Other pages of the Medical Information Form contain areas for recording details about respiratory function, cardiac function, feeding/nutrition, orthopaedic issues, eye issues, central nervous system issues, endocrine symptoms, pain, medications, allergies, general examination findings (including pulmonary function testing results) and neurologic examination findings - including areas for recoding details of the neuromuscular examination.

Simultaneous to my efforts to create data entry forms for the 'MD-CORE' initiative, I worked closely with other paediatric neuromuscular physicians from the United States in developing 'Core Data Elements' (CDEs) for the congenital muscular dystrophies, an effort launched by the National Institute of Neurological Disorders and Stroke (NINDS), National Institutes of Health (NIH), USA. The NINDS has promoted the creation of CDEs with the goal of facilitating clinical research as well as 'streamlining' data collection for clinical trials. These forms can be used for clinical research and clinical trials in any of the congenital muscular dystrophies and are accessible to clinical researchers and investigators online (http://www.commondataelements.ninds.nih.gov/CMD.aspx#tab=Data_Standards). Given my involvement in both the UK 'MD-CORE' and the USA CMD 'CDEs' initiatives, I have been able to help harmonise these natural history data collection tools. That is, the phenotypic data elements collected via the MD-CORE and the CDEs data entry forms largely overlap. In this manner, these data collection tools promote consistency between the natural history data gathered prior to clinical trials (which can serve as 'lead-in' data) and the natural history data gathered during potential clinical trials in the collagen VI-related myopathies as well as other CMD subtypes.

Detailed natural history data such as the extensive pulmonary function data collected in an international cohort of collagen VI-related myopathy patients (Chapter 2) promotes the optimisation of clinical care. Furthermore, natural history data is a prerequisite for effective clinical trial design. In particular, natural history data is essential for determining appropriate inclusive criteria, exclusion criteria and outcome measures in clinical trials. The delineation of annual rate of decline in forced vital capacity (FVC) in UCMD and intermediate collagen VI-related myopathy patients has identified pulmonary function as a disease-relevant outcome measure which is also viable, given the ability of FVC to be measured in patients regardless of degree of muscle weakness or joint contractures.

Given the wide phenotypic spectrum of collagen VI-related myopathies, finding motor scales which are effective in capturing the natural history of motor function in patients with varied clinical severity is challenging. Furthermore, the ability of a particular motor scale to serve as an outcome measure in clinical trials depends on its ability to be relevant to the patient's motor phenotype as well as its ability to measure change within the timeframe of a potential clinical trial.[256] As a member of the Congenital Muscular Dystrophy Outcome Measures Working Group (jointly coordinated and

sponsored by the NINDS and Cure CMD, a non-profit patient organisation), I collaborated with neuromuscular specialists and physiotherapists from neuromuscular centres in Europe, the United States and Australia in an effort to establish outcome measure candidates for the congenital muscular dystrophies. Along with other members of this CMD Outcome Measures Working Group, I assessed collagen VI-related myopathy patients as well as merosin-deficient CMD (MDC1A) patients during annual Outcome Measure Study weekends when we trialled various pulmonary function tests and motor scales in CMD patients who had consented to participate in this NINDS sponsored study.

In particular, I piloted a motor function scale called the Egen Klassifikation (EK) (Danish for 'our scale') on a total of 29 CMD patients. The EK scale was created in Denmark for assessing motor function in non-ambulant Duchenne muscular dystrophy patients (Figure 26).[257] The validity of this scale's sum score in distinguishing differences in muscle strength was demonstrated in a study of 56 Duchenne muscular dystrophy and 38 spinal muscular atrophy patients.[257] A small pilot of the EK scale version 2 (EK2) scale in eight non-ambulant CMD patients (Newcastle, UK) found that the majority of the EK2 scale seemed relevant to CMD patients.[256] I led an extended pilot of the EK2 scale by using this scale to assess collagen VI-related myopathy and MDC1A patients. Following the first NINDS CMD Outcome Measures Study (2010) when I evaluated 21 patients with collagen VI-related myopathy or merosin deficient congenital muscular dystrophy using the EK2 scale, I collaborated with a UK neuromuscular physiotherapist (Dr Anna Mayhew, Institute of Human Genetics, International Centre for Life, University of Newcastle, UK) and a Danish neuromuscular physiotherapist scale (Dr Birgit Steffensen, National Rehabilitation Centre for Neuromuscular Diseases, Aarhus, Denmark)- the original author of the EK scale- in adapting the EK2 scale for CMD patients, in particular to better capture hand function (Figure 27). I then repeated evaluations of the same 21 CMD patients (as well as 8 others, for a total of 29 CMD patients) using the EK2 scale (the original version as well as the version adapted for use in CMD patients) at the one year follow-up NIH CMD Outcome Measures Study (2011). The analysis of this data remains in progress as part of an ongoing NINDS CMD Outcome Measures Study. The results of this study will help to inform clinical trial planning and also provide further natural history data for the collagen VI-related myopathy and MDC1A patient populations.

Egen Klassifikation Scale Version 2 (EK2) **Patient Letter: _____**

Steffensen 2008

Name **DOB**

Date of assessment

Date of spinal surgery **Assessor** (please circle)

NOTE: *Score the best you have done in the last two weeks especially if there is variation between good and bad days

1	**Ability to use wheelchair** How do you get around indoors and outdoors?	N/A
	Able to use a manual wheelchair on flat ground, 10m < 1 minute	0
	Able to use a manual wheelchair on flat ground, 10m > 1 minute	1
	Unable to use manual wheelchair, requires power wheelchair	2
	Uses power wheelchair, but occasionally has difficulty steering	3

2	**Ability to transfer from wheelchair** How do you transfer from your wheelchair to a bed?	N/A
	Able to transfer from wheelchair without help	0
	Able to transfer independently from wheelchair, with use of aid	1
	Needs assistance to transfer with or without additional aids (hoist, easy glide)	2
	Needs to be lifted with support of head when transferring from wheelchair	3

3	**Ability to stand** Do you sometimes stand? How do you do this?	N/A
	Able to stand with knees supported, as when using braces	0
	Able to stand with knees and hips supported, as when using standing aids	1
	Able to stand with full body support	2
	Unable to be stood	3

4	**Ability to balance in the wheelchair** Can you bend forwards and to the sides and return to the upright position?	N/A
	Able to push himself upright from complete forward flexion by pushing up with hands	0
	Able to move the upper part of the body > 30 in all directions from the upright position, but cannot push himself upright as above	1
	Able to move the upper part of the body < 30 from one side to the other	2
	Unable to change position of the upper part of the body, cannot sit without total support of the trunk and head	3

5	**Ability to move the arms** Can you move your fingers, hands and arms against gravity?	N/A
	Able to raise the arms above the head with or without compensatory movements	0
	Unable to lift the arms above the head, but able to raise the forearms against gravity, ie. hand to mouth with / without elbow support	1
	Unable to lift the forearms against gravity, but able to use the hands against gravity when the forearm is supported	2
	Unable to move the hands against gravity but able to use the fingers	3

6	**Ability to use the hands and arms for eating** Can you describe how you eat?	N/A
	Able to eat and drink without elbow support	0
	Eats or drinks with support at elbow	1
	Eats and drinks with elbow support; with reinforcement of the opposite hand +or – aids	2
	Has to be fed	3

7	**Ability to turn in bed** How do you turn in bed during the night?	N/A
	Able to turn himself in bed with bedclothes	0
	Needs some help to turn in bed or can turn in some directions	1
	Unable to turn himself in bed. Has to be turned 0 - 3 times during the night	2
	Unable to turn himself in bed. Has to be turned > 4 times during the night	3

8	**Ability to cough** How do you cough when you have to?	N/A
	Able to cough effectively	0
	Has difficulty to cough and sometimes needs manual reinforcement. Able to clear throat	1
	Always needs help with coughing. Only possible to cough in certain positions	2
	Unable to cough, Needs suction and/or hyperventilation techniques or IPPB in order to keep airways clear	3

9	**Ability to speak** Can you speak so that what you say can be understood if you sit at the back of a large room?	N/A
	Powerful speech. Able to sing and speak loudly	0
	Speaks normally, but cannot raise his voice	1
	Speaks with quiet voice and needs a breath after 3 to 5 words	2
	Speech is difficult to understand except to close relatives	3

10	**Physical well-being** (This relates to respiratory insufficiency only – see manual)	N/A
	No complaints, feels good	0
	Easily tires. Has difficulty resting in a chair or in bed	1
	Has loss of weight, loss of appetite, Scared of falling asleep at night, sleeps badly	2
	Experience additional symptoms: change of mood, stomach ache, palpitations, perspiring,	3

11	**Daytime fatigue** Do you have to organise your day or take a rest to avoid getting too tired?	N/A
	Doesn't get tired during day	0
	Need to limit activity to avoid getting too tired	1
	Need to limit my activity **and** have a rest period to avoid getting too tired	2
	Get tired during day even if I rest and limit activity	3

12	**Head Control** How much head support do you need in your wheelchair?	N/A
	Does not need head support	0
	Needs head support when going up and down slope (15° standard ramp)	1
	Needs head support when driving wheelchair	2
	When sitting still in a wheelchair needs head support	3

13	**Ability to control Joystick** What kind of joystick do you use to control your chair?	N/A
	Uses a standard joystick without special adaptation	0
	Uses an adapted joystick or has adjusted wheelchair in order to use joystick	1
	Uses other techniques for steering than joystick such as blowing sucking systems or scanned driving	2
	Unable to operate wheelchair. Needs another person to operate it	3

14	**Food Textures** Do you have to modify your food in any way in order to eat it?	N/A
	Eats all textures of food	0
	Eats cut up / chunky food or avoids hard/chewy foods	1
	Eats minced/ pureed food with supplementation as required	2
	Main intake consists of being tube fed	3

15	**Eating a meal** (with or without assistance) How long does it take to complete a meal?	N/A
	Able to consume a whole meal in the same time as others sharing the meal	0
	Able to consume a whole meal in the same time as others only with encouragement or needs some additional time (approx 10 min)	1
	Able to consume a whole meal but requires substantially more time compared to others eating the same meal (15 m or more extra)	2
	Unable to consume a whole meal	3

16	**Swallowing** Do you ever have problems with swallowing?	N/A
	Never has problems when swallowing and never chokes on food/drink,	0
	May experience occasional (less than once a month) problems swallowing certain types of food or occasionally chokes	1
	Has regular trouble swallowing food/drink or chokes on food/drink (more than once a month)	2
	Has trouble swallowing saliva or secretions	3

17	**Hand function** Which of these activities can you do?	N/A
	Can unscrew the lid of a water of fizzy drink bottle and break the seal	0
	Can write two lines **or** use computer keyboard	1
	Can write signature **or** send text or use remote control	2
	Cannot use hands	3

	TOTAL SCORE / 51	

	Comments

Figure 26: EK2 scale recording form.

(EK2 scale items from Steffensen, Hyde, Lyager and Mattsson, 2001[257] and inclusive of further additions from Dr Birgit Steffensen, 2008.)

EK2 17a: Hand function	Can you do the following items using your hands?	**0: Can unscrew the lid of a water or fizzy drink and break the seal** **1: Can write two lines or use a computer keyboard (able to move arms)** **2: Can write signature or send text using remote control** **3: Cannot use hands**
EK2 17b: Hand function	How do you open up a water bottle?	**0: Can break seal and unscrew lid of water bottle** **1: Can unscrew lid once seal is broken** **2: Can remove lid unscrewed and resting on top of bottle** **3: Not able to remove lid**

Figure 27: Adaptations made to EK2 scale for use in congenital muscular dystrophy patients.

In particular, item 17 is divided into 2 parts (17a and 17b) in order to better capture hand function/ motor ability.

6.2 NAVIGATING THROUGH THE MOLECULAR MAZE AND MYOMATRIX

At present, the mechanisms by which a deficiency of the extracellular matrix protein collagen VI results in abnormal functioning of the mitochondrial permeability transition pore, increased apoptosis and aberrant autophagy have not been fully elucidated. Our understanding of the pathophysiologic mechanisms underlying collagen VI deficiency may increase, however, through the use of new genetic technologies. Indeed, the advent of next-generation/high-throughput sequencing has increased the potential for uncovering new genes in collagen VI-related myopathies, which could lead to increased understanding of molecular and biochemical pathways underlying the pathogenesis of collagen VI deficiency, thereby uncovering new therapeutic targets.

Our present understanding of the molecular mechanisms resulting from mutations in *COL6A1, COL6A2* and *COL6A3* may offer potential candidates for therapeutic targets, as well. In collagen VI-related myopathy due to autosomal recessive mutations in *COL6A1, COL6A2* or *COL6A3* and resulting in an absence of one of the collagen VI chains, a potential therapeutic strategy is gene replacement (of the mutated gene). Given the common mutational mechanism of *de novo* autosomal dominant mutations in the collagen VI-related myopathies, however, the potential use of this strategy would be limited. A strategy of functional knock-down or inactivation of the mutated allele may be possible in patients with *de novo* autosomal dominant collagen VI mutations,[258] especially given the evidence that haploinsufficiency for collagen VI genes does not result in neuromuscular disease (section 4.1).[152]

Our understanding of the interactions of collagen VI with its myomatrix neighbours may also inform therapeutic strategies. In particular, strategies aimed at stabilising the extracellular matrix (ECM) and/or the ECM–cytoskeletal link may be effective in stabilising sarcolemmal integrity and ultimately muscle function. While the potential of transgenic overexpression of integrin α7 of improving muscle pathology and function in collagen VI deficient muscle seems uncertain, given the preliminary evidence of upregulation of integrin α7 in collagen VI deficient patient muscle (section 5.2.6), the success of this strategy in the merosin deficient mouse model offers experimental evidence that this type of approach may be efficacious. Further studies of overexpression of other ECM proteins including biglycan will be necessary to determine if such an approach offers therapeutic promise by stabilising or improving muscle function in the collagen VI-related myopathies.

At present, the therapeutic target most extensively studied by groups working with the collagen VI-deficient animal models has been apoptosis. Studies both in the *Col6a1*$^{-/-}$ mouse as well as in collagen VI zebrafish models have revealed evidence of increased apoptosis[30, 89-92, 94] with improvement (normalisation of apoptosis) following treatment with cyclosporine A[30, 89-90, 94] or D-MeAla^3-EtVal^4-cyclosporin (Debio 025)[92] (section 1.5).

A further anti-apoptotic compound N-(dibenz(b,f)oxepin-10-ylmethyl)-N-methyl-N-prop-2-ynylamine maleate (Omigapil) has been studied in the merosin-deficient mouse model (*dy*^{w}/ *dy*^{w} mouse) where it demonstrated efficacy in inhibiting GAPDH-Siah1-mediated apoptosis in muscle with concomitant decreased weight loss and improved locomotor activity.[259] Ongoing studies of Omigapil in the *Col6a1*$^{-/-}$ mouse model have demonstrated efficacy in decreasing apoptosis in particular of the diaphragm muscle as well as improving skeletal muscle mitochondrial integrity (unpublished data).

Finally, when considering potential therapeutic targets in collagen VI-related myopathies, it is important to acknowledge emerging targets which may prove to be venues for future development. It is notable that while a deficiency of the collagen VI protein results in pathology in skeletal muscle, this is not the case in cardiac muscle. Indeed, cardiac involvement has not been documented in collagen VI-related myopathy patients (except for right-heart failure associated with untreated respiratory insufficiency). For this reason, it seems plausible that there are pathomechanisms underlying collagen VI-related myopathies which are specific to skeletal muscle. One possible explanation for why the heart appears unaffected by collagen VI deficiency is that cardiac muscle, unlike skeletal muscle, lacks satellite cells or analogous progenitor cells. Perhaps the pathophysiology of collagen VI deficiency is, in some part, related to problematic regeneration, for which therapeutic strategies focussed on muscle regeneration may be effective. In particular, recent research performed by colleagues at our neuromuscular centre (the Dubowitz Neuromuscular Centre) using human synovial stem cells (a subset of mesenchymal stem cells reported to regenerate muscle fibres and increase the satellite cell pool[260]) revealed that these stem cells secreted collagen VI and laminin α2 into the skeletal muscle (in an immunodeficient mouse model).[261] Further studies, and in particular studies performed in collagen VI-deficient animal models, would be necessary, however, to determine whether synovial stem cells may have therapeutic potential in collagen VI-related myopathies.

6.3 ON A JOURNEY TOWARD CLINICAL TRIALS

While experimental therapeutic approaches may offer promise in animal models, the real test of efficacy of these approaches lies in their ability to be translated to patients in clinical trials. Ultimately, a combination of various therapeutic approaches may prove to be the most efficacious in collagen VI-related myopathy patients; however, each therapeutic approach will need to be tested in isolation. Plans are presently being made for an experimental clinical trial of the anti-apoptotic compound Omigapil in patients with collagen VI-related myopathies as well as patients with merosin-deficient CMD, making the goal of preparing the collagen VI-related myopathy patient population for clinical trials more important than even. As apoptosis is an apparent common 'downstream' or secondary effect in skeletal muscles of both collagen VI deficiency and merosin deficiency patients, both collagen VI-related myopathy and merosin-deficient CMD patients are being considered for inclusion in an upcoming clinical trial to establish the safety and pharmacokinetics of Omigapil. [The safety of this compound in adults has been established from its use (under the name TCH346) in two separate clinical trials performed in neurodegenerative conditions with underlying apoptotic pathomechanisms: Parkinson's disease[262] and amyotrophic lateral sclerosis (ALS).[263] Unfortunately, this compound did not demonstrate efficacy when compared to placebo in either of those clinical trials.[262-263]]

I wrote the initial draft for a clinical trial of Omigapil in collagen VI-related myopathy patients via my participation in a Clinical Trial Methods Course in Neurology, organised and sponsored by the NINDS. This draft was used to write the present clinical trial protocol for Omigapil in collagen VI-related myopathies and merosin-deficient CMD, which is being planned for two study sites: the Great Ormond Street Hospital (London) and the National Institute of Neurological Disorders and Stroke/National Institutes of Health (Bethesda, Maryland, USA). The pulmonary function natural history data collected and analysed in collagen VI-related myopathy patients (Chapter 2) has been instrumental in informing the design of this clinical trial, which includes forced vital capacity as an outcome measure (to be trialled during the initial safety and pharmacokinetics study). While it remains to be seen whether Omigapil will result in clinical improvement in respiratory function and/or motor function for CMD patients with collagen VI deficiency or merosin deficiency, the planning of this first clinic trial for congenital muscular dystrophy patients, represents an important step forward for these rare disease patient populations.

The availability of next generation/high-throughput sequencing increases our potential for identifying new therapeutic targets in the collagen VI-related myopathies. It is important to remember that the process of translating research of any therapeutic target from the basic science lab -or the proverbial 'bench'- to clinical trials -or the proverbial 'bedside'- relies on well-defined patient populations including detailed knowledge of natural history and clearly defined phenotypic categories in target patient populations. Certainly, the planning of a clinical trial in the collagen VI-related myopathies and merosin-deficient CMD subgroups establishes an important precedent for the congenital muscular dystrophies, demonstrating that despite great challenges in gathering natural history data and identifying relevant and viable outcome measures in these rare diseases, these patient populations are indeed progressing forward toward clinical trials.

The journey toward clinical trials begins where it ends, with the patient. The clinical research discussed in this thesis has aimed to help improve that journey by navigating through the clinical manifestations (Chapters 2 and 3), the molecular maze (Chapter 4) and the myomatrix (Chapter 5). It is my hope that this clinical research, in particular the natural history data analysed, the diagnostic algorithms proposed and the tools created for ongoing natural history data collection, collectively proves to be effective in the continued journey of collagen VI-related myopathy patients towards future clinical trials.

REFERENCES

1. Batten F. Case of myositis fibrosa with pathological examination. Trans Clin Soc Lond 1904;37:12-22.
2. Mostacciuolo ML, Miorin M, Martinello F, Angelini C, Perini P, Trevisan CP. Genetic epidemiology of congenital muscular dystrophy in a sample from north-east Italy. Hum Genet 1996;97:277-9.
3. Darin N, Tulinius M. Neuromuscular disorders in childhood: a descriptive epidemiological study from western Sweden. Neuromuscul Disord 2000;10:1-9.
4. Mercuri E, Yuva Y, Brown SC, et al. Collagen VI involvement in Ullrich syndrome: a clinical, genetic, and immunohistochemical study. Neurology 2002;58:1354-9.
5. Muntoni F, Voit T. The congenital muscular dystrophies in 2004: a century of exciting progress. Neuromuscul Disord 2004;14:635-49.
6. Okada M, Kawahara G, Noguchi S, et al. Primary collagen VI deficiency is the second most common congenital muscular dystrophy in Japan. Neurology 2007;69:1035-42.
7. Norwood FL, Harling C, Chinnery PF, Eagle M, Bushby K, Straub V. Prevalence of genetic muscle disease in Northern England: in-depth analysis of a muscle clinic population. Brain 2009;132:3175-86.
8. Peat RA, Smith JM, Compton AG, et al. Diagnosis and etiology of congenital muscular dystrophy. Neurology 2008;71:312-21.
9. Clement EM, Feng L, Mein R, et al. Relative frequency of congenital muscular dystrophy subtypes: analysis of the UK diagnostic service 2001-2008. Neuromuscul Disord 2012;22:522-7.
10. Heiskanen M, Saitta B, Palotie A, Chu ML. Head to tail organization of the human COL6A1 and COL6A2 genes by fiber-FISH. Genomics 1995;29:801-3.
11. Voermans NC, Bonnemann CG, Huijing PA, et al. Clinical and molecular overlap between myopathies and inherited connective tissue diseases. Neuromuscul Disord 2008;18:843-56.
12. Weil D, Mattei MG, Passage E, et al. Cloning and chromosomal localization of human genes encoding the three chains of type VI collagen. Am J Hum Genet 1988;42:435-45.
13. Timpl R, Chu ML. Microfibrillar collagen type VI. In: Mecham RP, ed. Extracellular matrix assembly and structure. Orlando: Academic Press; 1994:207-42.
14. Timpl R, Engel J. Type VI collagen. In: Mayne R, Burgeson R, eds. Structure and function of collagen types. Orlando, FL: Academic Press; 1987:105-43.
15. Fitzgerald J, Rich C, Zhou FH, Hansen U. Three novel collagen VI chains, alpha4(VI), alpha5(VI), and alpha6(VI). J Biol Chem 2008;283:20170-80.
16. Ball SG, Baldock C, Kielty CM, Shuttleworth CA. The role of the C1 and C2 a-domains in type VI collagen assembly. J Biol Chem 2001;276:7422-30.
17. Lamande SR, Morgelin M, Adams NE, Selan C, Allen JM. The C5 domain of the collagen VI alpha3(VI) chain is critical for extracellular microfibril formation and is present in the extracellular matrix of cultured cells. J Biol Chem 2006;281:16607-14.
18. Furthmayr H, Wiedemann H, Timpl R, Odermatt E, Engel J. Electron-microscopical approach to a structural model of intima collagen. Biochem J 1983;211:303-11.
19. Odermatt E, Risteli J, van Delden V, Timpl R. Structural diversity and domain composition of a unique collagenous fragment (intima collagen) obtained from human placenta. Biochem J 1983;211:295-302.
20. Chu ML, Conway D, Pan TC, et al. Amino acid sequence of the triple-helical domain of human collagen type VI. J Biol Chem 1988;263:18601-6.
21. Chu ML, Pan TC, Conway D, et al. Sequence analysis of alpha 1(VI) and alpha 2(VI) chains of human type VI collagen reveals internal triplication of globular domains similar to the A domains of von Willebrand factor and two alpha 2(VI) chain variants that differ in the carboxy terminus. Embo J 1989;8:1939-46.
22. Chu ML, Zhang RZ, Pan TC, et al. Mosaic structure of globular domains in the human type VI collagen alpha 3 chain: similarity to von Willebrand factor, fibronectin, actin, salivary proteins and aprotinin type protease inhibitors. Embo J 1990;9:385-93.
23. Engvall E, Hessle H, Klier G. Molecular assembly, secretion, and matrix deposition of type VI collagen. J Cell Biol 1986;102:703-10.
24. Lamande SR, Sigalas E, Pan TC, et al. The role of the alpha3(VI) chain in collagen VI assembly. Expression of an alpha3(VI) chain lacking N-terminal modules N10-N7 restores collagen VI assembly, secretion, and matrix deposition in an alpha3(VI)-deficient cell line. J Biol Chem 1998;273:7423-30.

25. Baldock C, Sherratt MJ, Shuttleworth CA, Kielty CM. The supramolecular organization of collagen VI microfibrils. J Mol Biol 2003;330:297-307.
26. Pan TC, Zhang RZ, Sudano DG, Marie SK, Bonnemann CG, Chu ML. New molecular mechanism for Ullrich congenital muscular dystrophy: a heterozygous in-frame deletion in the COL6A1 gene causes a severe phenotype. Am J Hum Genet 2003;73:355-69.
27. Ishikawa H, Sugie K, Murayama K, et al. Ullrich disease: Collagen VI deficiency: EM suggests a new basis for muscular weakness. Neurology 2002;59:920-3.
28. Ishikawa H, Sugie K, Murayama K, et al. Ullrich disease due to deficiency of collagen VI in the sarcolemma. Neurology 2004;62:620-3.
29. Niiyama T, Higuchi I, Suehara M, et al. Electron microscopic abnormalities of skeletal muscle in patients with collagen VI deficiency in Ullrich's disease. Acta Neuropathol (Berl) 2002;104:67-71. Epub 2002 Apr 30.
30. Irwin WA, Bergamin N, Sabatelli P, et al. Mitochondrial dysfunction and apoptosis in myopathic mice with collagen VI deficiency. Nat Genet 2003;35:367-71.
31. Jimenez-Mallebrera C, Brown SC, Sewry CA, Muntoni F. Congenital muscular dystrophy: molecular and cellular aspects. Cell Mol Life Sci 2005;62:809-23.
32. Tillet E, Wiedemann H, Golbik R, et al. Recombinant expression and structural and binding properties of alpha 1(VI) and alpha 2(VI) chains of human collagen type VI [published erratum appears in Eur J Biochem 1994 Jun 15;222(3):1064]. European Journal of Biochemistry 1994;221:177-85.
33. Wiberg C, Hedbom E, Khairullina A, et al. Biglycan and decorin bind close to the n-terminal region of the collagen VI triple helix. J Biol Chem 2001;276:18947-52.
34. Bidanset DJ, Guidry C, Rosenberg LC, Choi HU, Timpl R, Hook M. Binding of the proteoglycan decorin to collagen type VI. J Biol Chem 1992;267:5250-6.
35. Kuo HJ, Maslen CL, Keene DR, Glanville RW. Type VI collagen anchors endothelial basement membranes by interacting with type IV collagen. Journal of Biological Chemistry 1997;272:26522-9.
36. Brown JC, Mann K, Wiedemann H, Timpl R. Structure and binding properties of collagen type XIV isolated from human placenta. J Cell Biol 1993;120:557-67.
37. Sasaki T, Gohring W, Pan TC, Chu ML, Timpl R. Binding of mouse and human fibulin-2 to extracellular matrix ligands. Journal of Molecular Biology 1995;254:892-9.
38. McDevitt CA, Marcelino J, Tucker L. Interaction of intact type VI collagen with hyaluronan. FEBS Lett 1991;294:167-70.
39. Kielty CM, Whittaker SP, Grant ME, Shuttleworth CA. Type VI collagen microfibrils: evidence for a structural association with hyaluronan. J Cell Biol 1992;118:979-90.
40. Specks U, Mayer U, Nischt R, et al. Structure of recombinant N-terminal globule of type VI collagen alpha 3 chain and its binding to heparin and hyaluronan. Embo J 1992;11:4281-90.
41. Finnis ML, Gibson MA. Microfibril-associated glycoprotein-1 (MAGP-1) binds to the pepsin-resistant domain of the alpha3(VI) chain of type VI collagen. J Biol Chem 1997;272:22817-23.
42. Stallcup WB, Dahlin K, Healy P. Interaction of the NG2 chondroitin sulfate proteoglycan with type VI collagen. J Cell Biol 1990;111:3177-88.
43. Bertini E, Pepe G. Collagen type VI and related disorders: Bethlem myopathy and Ullrich scleroatonic muscular dystrophy. Eur J Paediatr Neurol 2002;6:193-8.
44. Ullrich O. Kongenitale atonisch-sklerotische Muskeldystrophie. Monatsschr Kinderheilkd 1930;47:502-10.
45. Ullrich O. Kongenitale atonisch-sklerotische Muskeldystrophie, ein weiterer Typus der heredodegeneration Erkrankungen des neuromuskulären Systems. Z Ges Neurol Psychiat 1930;126:171-20.
46. Camacho Vanegas O, Bertini E, Zhang RZ, et al. Ullrich scleroatonic muscular dystrophy is caused by recessive mutations in collagen type VI. Proc Natl Acad Sci U S A 2001;98:7516-21.
47. Furukawa T, Toyokura Y. Congenital, hypotonic-sclerotic muscular dystrophy. J Med Genet 1977;14:426-9.
48. Nonaka I, Une Y, Ishihara T, Miyoshino S, Nakashima T, Sugita H. A clinical and histological study of Ullrich's disease (congenital atonic-sclerotic muscular dystrophy). Neuropediatrics 1981;12:197-208.
49. Lampe AK, Bushby KM. Collagen VI related muscle disorders. J Med Genet 2005;42:673-85.
50. Voit T. Congenital muscular dystrophies: 1997 update. Brain Dev 1998;20:65-74.
51. Bonnemann CG. The collagen VI-related myopathies: muscle meets its matrix. Nat Rev Neurol 2011;7:379-90.
52. Nadeau A, Kinali M, Main M, et al. Natural history of Ullrich congenital muscular dystrophy. Neurology 2009;73:25-31.

53. Allamand V, Merlini L, Bushby K. 166th ENMC International Workshop on Collagen type VI-related Myopathies, 22-24 May 2009, Naarden, The Netherlands. Neuromuscul Disord 2010;20:346-54.
54. Brinas L, Richard P, Quijano-Roy S, et al. Early onset collagen VI myopathies: Genetic and clinical correlations. Ann Neurol 2010;68:511-20.
55. Bethlem J, G.K. vW. Benign myopathy, wth autosomal dominant inheritance: a report on three pedigrees. Brain 1976;99:91-100.
56. Jöbsis GJ, Bolhuis PA, Boers JM, et al. Genetic localization of Bethlem myopathy. Neurology 1996;46:779-82.
57. Jobsis GJ, Keizers H, Vreijling JP, et al. Type VI collagen mutations in Bethlem myopathy, an autosomal dominant myopathy with contractures. Nat Genet 1996;14:113-5.
58. Pan TC, Zhang RZ, Pericak-Vance MA, et al. Missense mutation in a von Willebrand factor type A domain of the alpha 3(VI) collagen gene (COL6A3) in a family with Bethlem myopathy. Hum Mol Genet 1998;7:807-12.
59. Haq RU, Speer MC, Chu ML, Tandan R. Respiratory muscle involvement in Bethlem myopathy. Neurology 1999;52:174-6.
60. Foley AR, Hu Y, Zou Y, et al. Autosomal recessive inheritance of classic Bethlem myopathy. Neuromuscul Disord 2009.
61. Gualandi F, Urciuolo A, Martoni E, et al. Autosomal recessive Bethlem myopathy. Neurology 2009;73:1883-91.
62. Merlini L, Martoni E, Grumati P, et al. Autosomal recessive myosclerosis myopathy is a collagen VI disorder. Neurology 2008;71:1245-53.
63. Jöbsis GJ, Boers JM, Barth PG, de Visser M. Bethlem myopathy: a slowly progressive congenital muscular dystrophy with contractures. Brain 1999;122:649-55.
64. Mohire MD, Tandan R, Fries TJ, Little BW, Pendlebury WW, Bradley WG. Early-onset benign autosomal dominant limb-girdle myopathy with contractures (Bethlem myopathy). Neurology 1988;38:573-80.
65. Pepe G, de Visser M, Bertini E, et al. Bethlem Myopathy (BETHLEM): 86th ENMC international workshop, 10-11 November 2000, Naarden, The Netherlands. Neuromuscular Disorders 2002;12:296-305.
66. Nadeau A, Muntoni F. Skin changes in Ullrich congenital muscular dystrophy. Neuromuscul Disord 2008;18:982.
67. Collins J, Foley AR, Straub V, Bonnemann CG. Spontaneous keloid formation in patients with Bethlem myopathy. Neurology 2012;79:2158.
68. Baker NL, Morgelin M, Peat R, et al. Dominant collagen VI mutations are a common cause of Ullrich congenital muscular dystrophy. Hum Mol Genet 2005;14:279-93.
69. Lampe AK, Zou Y, Sudano D, et al. Exon skipping mutations in collagen VI are common and are predictive for severity and inheritance. Hum Mutat 2008;29:809-22.
70. Baker NL, Morgelin M, Peat R, et al. Dominant collagen VI mutations are a common cause of Ullrich congenital muscular dystrophy. Hum Mol Genet 2004.
71. Pepe G, Giusti B, Bertini E, et al. A heterozygous splice site mutation in COL6A1 leading to an in-frame deletion of the alpha1(VI) collagen chain in an italian family affected by bethlem myopathy. Biochem Biophys Res Commun 1999;258:802-7.
72. Vanegas OC, Zhang RZ, Sabatelli P, et al. Novel COL6A1 splicing mutation in a family affected by mild Bethlem myopathy. Muscle Nerve 2002;25:513-9.
73. Lucioli S, Giusti B, Mercuri E, et al. Detection of common and private mutations in the COL6A1 gene of patients with Bethlem myopathy. Neurology 2005;64:1931-7.
74. Lampe AK, Dunn DM, von Niederhausern AC, et al. Automated genomic sequence analysis of the three collagen VI genes: applications to Ullrich congenital muscular dystrophy and Bethlem myopathy. J Med Genet 2005;42:108-20.
75. Pepe G, Lucarini L, Zhang RZ, et al. COL6A1 genomic deletions in Bethlem myopathy and Ullrich muscular dystrophy. Ann Neurol 2006;59:190-5.
76. Scacheri PC, Gillanders EM, Subramony SH, et al. Novel mutations in collagen VI genes: expansion of the Bethlem myopathy phenotype. Neurology 2002;58:593-602.
77. Pepe G, Bertini E, Giusti B, et al. A novel de novo mutation in the triple helix of the COL6A3 gene in a two-generation Italian family affected by Bethlem myopathy. A diagnostic approach in the mutations' screening of type VI collagen. Neuromuscul Disord 1999;9:264-71.
78. Pace RA, Peat RA, Baker NL, et al. Collagen VI glycine mutations: perturbed assembly and a spectrum of clinical severity. Ann Neurol 2008;64:294-303.

79. Lucarini L, Giusti B, Zhang RZ, et al. A homozygous COL6A2 intron mutation causes in-frame triple-helical deletion and nonsense-mediated mRNA decay in a patient with Ullrich congenital muscular dystrophy. Hum Genet 2005;117:460-6. Epub 2005 Jun 17.
80. Zhang RZ, Zou Y, Pan TC, et al. Recessive COL6A2 C-globular missense mutations in Ullrich congenital muscular dystrophy: role of the C2a splice variant. J Biol Chem 2010;285:10005-15.
81. Brinas L, Richard P, Quijano-Roy S, et al. Early onset collagen VI myopathies: Genetic and clinical correlations. Ann Neurol 2010;68:511-20.
82. Ball S, Bella J, Kielty C, Shuttleworth A. Structural basis of type VI collagen dimer formation. J Biol Chem 2003;278:15326-32.
83. Tooley LD, Zamurs L, Beecher N, et al. Collagen VI microfibril formation is abolished by an {alpha}2(VI) von Willebrand factor type A domain mutation in a patient with Ullrich congenital muscular dystrophy. J Biol Chem 2010;285:33567-76.
84. Saitta B, Stokes DG, Vissing H, Timpl R, Chu ML. Alternative splicing of the human alpha 2(VI) collagen gene generates multiple mRNA transcripts which predict three protein variants with distinct carboxyl termini. J Biol Chem 1990;265:6473-80.
85. Gara SK, Grumati P, Urciuolo A, et al. Three novel collagen VI chains with high homology to the alpha3 chain. J Biol Chem 2008;283:10658-70.
86. Bonaldo P, Braghetta P, Zanetti M, Piccolo S, Volpin D, Bressan GM. Collagen VI deficiency induces early onset myopathy in the mouse: an animal model for Bethlem myopathy. Hum Mol Genet 1998;7:2135-40.
87. Izu Y, Ansorge HL, Zhang G, et al. Dysfunctional tendon collagen fibrillogenesis in collagen VI null mice. Matrix Biol 2011;30:53-61.
88. Christensen SE, Coles JM, Zelenski NA, et al. Altered trabecular bone structure and delayed cartilage degeneration in the knees of collagen VI null mice. PLoS One 2012;7:e33397.
89. Angelin A, Tiepolo T, Sabatelli P, et al. Mitochondrial dysfunction in the pathogenesis of Ullrich congenital muscular dystrophy and prospective therapy with cyclosporins. Proc Natl Acad Sci U S A 2007;104:991-6.
90. Angelin A, Bonaldo P, Bernardi P. Altered threshold of the mitochondrial permeability transition pore in Ullrich congenital muscular dystrophy. Biochim Biophys Acta 2008;1777:893-6.
91. Palma E, Tiepolo T, Angelin A, et al. Genetic ablation of cyclophilin D rescues mitochondrial defects and prevents muscle apoptosis in collagen VI myopathic mice. Hum Mol Genet 2009;18:2024-31.
92. Tiepolo T, Angelin A, Palma E, et al. The cyclophilin inhibitor Debio 025 normalizes mitochondrial function, muscle apoptosis and ultrastructural defects in Col6a1(-/-) myopathic mice. Br J Pharmacol 2009;157:1045-52.
93. Grumati P, Coletto L, Sabatelli P, et al. Autophagy is defective in collagen VI muscular dystrophies, and its reactivation rescues myofiber degeneration. Nat Med 2010;16:1313-20.
94. Telfer WR, Busta AS, Bonnemann CG, Feldman EL, Dowling JJ. Zebrafish models of collagen VI-related myopathies. Hum Mol Genet 2010;19:2433-44.
95. Wang CH, Bonnemann CG, Rutkowski A, et al. Consensus statement on standard of care for congenital muscular dystrophies. J Child Neurol 2010;25:1559-81.
96. Clement EM, Feng L, Mein R, et al. Relative frequency of congenital muscular dystrophy subtypes: Analysis of the UK diagnostic service 2001-2008. Neuromuscul Disord 2012.
97. Wallgren-Pettersson C, Bushby K, Mellies U, Simonds A. 117th ENMC workshop: ventilatory support in congenital neuromuscular disorders -- congenital myopathies, congenital muscular dystrophies, congenital myotonic dystrophy and SMA (II) 4-6 April 2003, Naarden, The Netherlands. Neuromuscul Disord 2004;14:56-69.
98. Nicot F, Hart N, Forin V, et al. Respiratory muscle testing: a valuable tool for children with neuromuscular disorders. Am J Respir Crit Care Med 2006;174:67-74.
99. Miller MR, Hankinson J, Brusasco V, et al. Standardisation of spirometry. Eur Respir J 2005;26:319-38.
100. Littell RC. SAS system for mixed models. Cary, N.C.: SAS Institute Inc.; 1996.
101. Vonesh EF, Chinchilli VM, Pu K. Goodness-of-fit in generalized nonlinear mixed-effects models. Biometrics 1996;52:572-87.
102. McMenamin JB, Becker LE, Murphy EG. Congenital muscular dystrophy: a clinicopathologic report of 24 cases. J Pediatr 1982;100:692-7.
103. Wang CH, Bonnemann CG, Rutkowski A, et al. Consensus statement on standard of care for congenital muscular dystrophies. J Child Neurol 2010;25:1559-81.
104. ATS/ERS Statement on respiratory muscle testing. Am J Respir Crit Care Med 2002;166:518-624.

105. Panitch HB. Diurnal hypercapnia in patients with neuromuscular disease. Paediatr Respir Rev 2010;11:3-8.
106. Smith PE, Calverley PM, Edwards RH, Evans GA, Campbell EJ. Practical problems in the respiratory care of patients with muscular dystrophy. N Engl J Med 1987;316:1197-205.
107. Laghi F, Tobin MJ. Disorders of the respiratory muscles. Am J Respir Crit Care Med 2003;168:10-48.
108. Misuri G, Lanini B, Gigliotti F, et al. Mechanism of CO(2) retention in patients with neuromuscular disease. Chest 2000;117:447-53.
109. Clinical indications for noninvasive positive pressure ventilation in chronic respiratory failure due to restrictive lung disease, COPD, and nocturnal hypoventilation--a consensus conference report. Chest 1999;116:521-34.
110. McCool FD, Tzelepis GE. Dysfunction of the diaphragm. N Engl J Med 2012;366:932-42.
111. Laroche CM, Carroll N, Moxham J, Green M. Clinical significance of severe isolated diaphragm weakness. Am Rev Respir Dis 1988;138:862-6.
112. Merlini L, Sabatelli P, Armaroli A, et al. Cyclosporine A in Ullrich congenital muscular dystrophy: long-term results. Oxid Med Cell Longev 2011;2011:139194.
113. Stanojevic S, Wade A, Cole TJ, et al. Spirometry centile charts for young Caucasian children: the Asthma UK Collaborative Initiative. Am J Respir Crit Care Med 2009;180:547-52.
114. Gauld LM, Kappers J, Carlin JB, Robertson CF. Prediction of childhood pulmonary function using ulna length. Am J Respir Crit Care Med 2003;168:804-9.
115. Ingelfinger JR, Drazen JM. Patient organizations and research on rare diseases. N Engl J Med 2011;364:1670-1.
116. Lagakos SW. Clinical trials and rare diseases. N Engl J Med 2003;348:2455-6.
117. Hicks D, Lampe AK, Barresi R, et al. A refined diagnostic algorithm for Bethlem myopathy. Neurology 2008;70:1192-9.
118. Jimenez-Mallebrera C, Maioli MA, Kim J, 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. Neuromuscul Disord 2006;16:571-82.
119. Zou Y, Zhang RZ, Sabatelli P, Chu ML, Bonnemann CG. Muscle Interstitial Fibroblasts Are the Main Source of Collagen VI Synthesis in Skeletal Muscle: Implications for Congenital Muscular Dystrophy Types Ullrich and Bethlem. J Neuropathol Exp Neurol 2008;67:144-54.
120. Muntoni F, Bertini E, Bonnemann C, et al. 98th ENMC International Workshop on Congenital Muscular Dystrophy (CMD), 7th Workshop of the International Consortium on CMD, 2nd Workshop of the MYO CLUSTER project GENRE. 26-28th October, 2001, Naarden, The Netherlands. Neuromuscul Disord 2002;12:889-96.
121. Higashi K, Higuchi I, Niiyama T, et al. Abnormal expression of proteoglycans in Ullrich's disease with collagen VI deficiency. Muscle Nerve 2006;33:120-6.
122. Giusti B, Lucarini L, Pietroni V, et al. Dominant and recessive COL6A1 mutations in Ullrich scleroatonic muscular dystrophy. Ann Neurol 2005;58:400-10.
123. Kim J, Jimenez-Mallebrera C, Foley AR, et al. Flow cytometry analysis: a quantitative method for collagen VI deficiency screening. Neuromuscul Disord 2012;22:139-48.
124. Mercuri E, Pichiecchio A, Counsell S, et al. A short protocol for muscle MRI in children with muscular dystrophies. Eur J Paediatr Neurol 2002;6:305-7.
125. Lamminen AE. Magnetic resonance imaging of primary skeletal muscle diseases: patterns of distribution and severity of involvement. Br J Radiol 1990;63:946-50.
126. Mercuri E, Cini C, Counsell S, et al. Muscle MRI findings in a three-generation family affected by Bethlem myopathy. Eur J Paediatr Neurol 2002;6:309-14.
127. Mercuri E, Cini C, Pichiecchio A, et al. Muscle magnetic resonance imaging in patients with congenital muscular dystrophy and Ullrich phenotype. Neuromuscul Disord 2003;13:554-8.
128. Mercuri E, Lampe A, Allsop J, et al. Muscle MRI in Ullrich congenital muscular dystrophy and Bethlem myopathy. Neuromuscul Disord 2005;15:303-10.
129. Mercuri E, Clements E, Offiah A, et al. Muscle magnetic resonance imaging involvement in muscular dystrophies with rigidity of the spine. Ann Neurol 2010;67:201-8.
130. Bonnemann CG, Brockmann K, Hanefeld F. Muscle ultrasound in bethlem myopathy. Neuropediatrics 2003;34:335-6.
131. Mercuri E, Lampe A, Allsop J, et al. Muscle MRI in Ullrich congenital muscular dystrophy and Bethlem myopathy. Neuromuscul Disord 2005;15:303-10.
132. Heckmatt JA, Dubowitz V, Leeman S. Detection of pathological change in dystrophic muscle with B-scan ultrasound imaging. Lancet 1980;1:1389-90.

133. Heckmatt JZ, Leeman S, Dubowitz V. Ultrasound imaging in the diagnosis of muscle disease. J Pediatr 1982;101:656-60.
134. Heckmatt JZ, Pier N, Dubowitz V. Real-time ultrasound imaging in muscles. Muscle Nerve 1988;11:56-65.
135. Pillen S, Arts IM, Zwarts MJ. Muscle ultrasound in neuromuscular disorders. Muscle Nerve 2008;37:679-93.
136. Zuberi SM, Matta N, Nawaz S, Stephenson JB, McWilliam RC, Hollman A. Muscle ultrasound in the assessment of suspected neuromuscular disease in childhood. Neuromuscul Disord 1999;9:203-7.
137. Drakonaki EE, Allen GM. Magnetic resonance imaging, ultrasound and real-time ultrasound elastography of the thigh muscles in congenital muscle dystrophy. Skeletal Radiol 2010;39:391-6.
138. Brockmann K, Becker P, Schreiber G, Neubert K, Brunner E, Bonnemann C. Sensitivity and specificity of qualitative muscle ultrasound in assessment of suspected neuromuscular disease in childhood. Neuromuscul Disord 2007;17:517-23.
139. Mercuri E. An integrated approach to the diagnosis of muscle disorders: what is the role of muscle imaging? Dev Med Child Neurol 2010;52:693.
140. Flanigan KM, Kerr L, Bromberg MB, et al. Congenital muscular dystrophy with rigid spine syndrome: a clinical, pathological, radiological, and genetic study. Ann Neurol 2000;47:152-61.
141. Mercuri E, Talim B, Moghadaszadeh B, et al. Clinical and imaging findings in six cases of congenital muscular dystrophy with rigid spine syndrome linked to chromosome 1p (RSMD1). Neuromuscul Disord 2002;12:631-8.
142. Mercuri E, Counsell S, Allsop J, et al. Selective muscle involvement on magnetic resonance imaging in autosomal dominant Emery-Dreifuss muscular dystrophy. Neuropediatrics 2002;33:10-4.
143. Carboni N, Mura M, Marrosu G, et al. Muscle MRI findings in patients with an apparently exclusive cardiac phenotype due to a novel LMNA gene mutation. Neuromuscul Disord 2008;18:291-8.
144. Bonne G, Yaou RB, Beroud C, et al. 108th ENMC International Workshop, 3rd Workshop of the MYO-CLUSTER project: EUROMEN, 7th International Emery-Dreifuss Muscular Dystrophy (EDMD) Workshop, 13-15 September 2002, Naarden, The Netherlands. Neuromuscul Disord 2003;13:508-15.
145. Quijano-Roy S, Mbieleu B, Bonnemann CG, et al. De novo LMNA mutations cause a new form of congenital muscular dystrophy. Ann Neurol 2008;64:177-86.
146. Voermans NC, Bonnemann CG, Hamel BC, Jungbluth H, van Engelen BG. Joint hypermobility as a distinctive feature in the differential diagnosis of myopathies. J Neurol 2009;256:13-27.
147. Voermans NC, van Alfen N, Pillen S, et al. Neuromuscular involvement in various types of Ehlers-Danlos syndrome. Ann Neurol 2009;65:687-97.
148. Mein R. GSTS Pathology DNA Laboratory Standard Operating Procedure: Congenital Muscular Dystrophy Referral Criteria. In. London; 2010:1-8.
149. Allamand V, Merlini L, Bushby K, Myopathies CfCV-R. 166th ENMC International Workshop on Collagen type VI-related Myopathies, 22-24 May 2009, Narden, The Netherlands. Neuromuscul Disord 2010;20:346-54.
150. Petrini S, D'Amico A, Sale P, et al. Ullrich myopathy phenotype with secondary ColVI defect identified by confocal imaging and electron microscopy analysis. Neuromuscul Disord 2007;17:587-96.
151. Strachan T, Read AP. Human molecular genetics. 4th ed. New York: Garland Science; 2011.
152. Foley AR, Hu Y, Zhou Y, et al. Large genomic deletions: a novel cause of Ullrich congenital muscular dystrophy. Ann Neurol 2011;69:206-11.
153. Lamande SR, Bateman JF, Hutchison W, et al. Reduced collagen VI causes Bethlem myopathy: a heterozygous COL6A1 nonsense mutation results in mRNA decay and functional haploinsufficiency. Hum Mol Genet 1998;7:981-9.
154. Phimister EG, Feero WG, Guttmacher AE. Realizing genomic medicine. N Engl J Med 2012;366:757-9.
155. Lupski JR, Reid JG, Gonzaga-Jauregui C, et al. Whole-genome sequencing in a patient with Charcot-Marie-Tooth neuropathy. N Engl J Med 2010;362:1181-91.
156. Komar AA. Single nucleotide polymorphisms : methods and protocols. 2nd ed. New York: Humana; 2009.
157. Saitta B, Timpl R, Chu ML. Human alpha 2(VI) collagen gene. Heterogeneity at the 5'-untranslated region generated by an alternate exon. J Biol Chem 1992;267:6188-96.

158. Sulonen AM, Ellonen P, Almusa H, et al. Comparison of solution-based exome capture methods for next generation sequencing. Genome Biol 2011;12:R94.
159. Ku CS, Cooper DN, Polychronakos C, Naidoo N, Wu M, Soong R. Exome sequencing: dual role as a discovery and diagnostic tool. Ann Neurol 2012;71:5-14.
160. Hoischen A, Gilissen C, Arts P, et al. Massively parallel sequencing of ataxia genes after array-based enrichment. Hum Mutat 2010;31:494-9.
161. Kozarewa I, Ning Z, Quail MA, Sanders MJ, Berriman M, Turner DJ. Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of (G+C)-biased genomes. Nat Methods 2009;6:291-5.
162. Aird D, Ross MG, Chen WS, et al. Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries. Genome Biol 2011;12:R18.
163. Benjamini Y, Speed TP. Summarizing and correcting the GC content bias in high-throughput sequencing. Nucleic Acids Res 2012;40:e72.
164. Ng SB, Turner EH, Robertson PD, et al. Targeted capture and massively parallel sequencing of 12 human exomes. Nature 2009;461:272-6.
165. Ng SB, Buckingham KJ, Lee C, et al. Exome sequencing identifies the cause of a mendelian disorder. Nat Genet 2010;42:30-5.
166. Sundaram SK, Huq AM, Sun Z, et al. Exome sequencing of a pedigree with Tourette syndrome or chronic tic disorder. Ann Neurol 2011;69:901-4.
167. Ng SB, Nickerson DA, Bamshad MJ, Shendure J. Massively parallel sequencing and rare disease. Hum Mol Genet 2010;19:R119-24.
168. Ku CS, Naidoo N, Pawitan Y. Revisiting Mendelian disorders through exome sequencing. Hum Genet 2011;129:351-70.
169. Majewski J, Schwartzentruber J, Lalonde E, Montpetit A, Jabado N. What can exome sequencing do for you? J Med Genet 2011;48:580-9.
170. Teer JK, Mullikin JC. Exome sequencing: the sweet spot before whole genomes. Hum Mol Genet 2010;19:R145-51.
171. Choi M, Scholl UI, Ji W, et al. Genetic diagnosis by whole exome capture and massively parallel DNA sequencing. Proc Natl Acad Sci U S A 2009;106:19096-101.
172. Bonnefond A, Durand E, Sand O, et al. Molecular diagnosis of neonatal diabetes mellitus using next-generation sequencing of the whole exome. PLoS One 2010;5:e13630.
173. Montenegro G, Powell E, Huang J, et al. Exome sequencing allows for rapid gene identification in a Charcot-Marie-Tooth family. Ann Neurol 2011;69:464-70.
174. Ng SB, Bigham AW, Buckingham KJ, et al. Exome sequencing identifies MLL2 mutations as a cause of Kabuki syndrome. Nat Genet 2010;42:790-3.
175. Ostergaard P, Simpson MA, Brice G, et al. Rapid identification of mutations in GJC2 in primary lymphoedema using whole exome sequencing combined with linkage analysis with delineation of the phenotype. J Med Genet 2011;48:251-5.
176. Sirmaci A, Walsh T, Akay H, et al. MASP1 mutations in patients with facial, umbilical, coccygeal, and auditory findings of Carnevale, Malpuech, OSA, and Michels syndromes. Am J Hum Genet 2010;87:679-86.
177. Walsh T, Shahin H, Elkan-Miller T, et al. Whole exome sequencing and homozygosity mapping identify mutation in the cell polarity protein GPSM2 as the cause of nonsyndromic hearing loss DFNB82. Am J Hum Genet 2010;87:90-4.
178. Wang JL, Yang X, Xia K, et al. TGM6 identified as a novel causative gene of spinocerebellar ataxias using exome sequencing. Brain 2010;133:3510-8.
179. Puffenberger EG, Jinks RN, Sougnez C, et al. Genetic mapping and exome sequencing identify variants associated with five novel diseases. PLoS One 2012;7:e28936.
180. Foo JN, Liu JJ, Tan EK. Whole-genome and whole-exome sequencing in neurological diseases. Nat Rev Neurol 2012.
181. Bilguvar K, Ozturk AK, Louvi A, et al. Whole-exome sequencing identifies recessive WDR62 mutations in severe brain malformations. Nature 2010;467:207-10.
182. Hanchard NA, Murdock DR, Magoulas PL, et al. Exploring the utility of Whole-Exome Sequencing as a diagnostic tool in a child with Atypical Episodic Muscle Weakness. Clin Genet 2012.
183. Rosewich H, Thiele H, Ohlenbusch A, et al. Heterozygous de-novo mutations in ATP1A3 in patients with alternating hemiplegia of childhood: a whole-exome sequencing gene-identification study. Lancet Neurol 2012;11:764-73.
184. Hamdan FF, Daoud H, Patry L, et al. Parent-child exome sequencing identifiesa de novo truncating mutation in TCF4 in non-syndromic intellectual disability. Clin Genet 2012.

185. Green ED, Guyer MS. Charting a course for genomic medicine from base pairs to bedside. Nature 2011;470:204-13.
186. Tong MY, Cassa CA, Kohane IS. Automated validation of genetic variants from large databases: ensuring that variant references refer to the same genomic locations. Bioinformatics 2011;27:891-3.
187. Richards CS, Bale S, Bellissimo DB, et al. ACMG recommendations for standards for interpretation and reporting of sequence variations: Revisions 2007. Genet Med 2008;10:294-300.
188. Dubowitz V, Sewry CA, eds. Muscle biopsy: A practical approach. 3rd ed. London: Elsevier; 2007.
189. Higuchi I, Horikiri T, Niiyama T, et al. Pathological characteristics of skeletal muscle in Ullrich's disease with collagen VI deficiency. Neuromuscul Disord 2003;13:310-6.
190. Schessl J, Goemans NM, Magold AI, et al. Predominant fiber atrophy and fiber type disproportion in early ullrich disease. Muscle Nerve 2008;38:1184-91.
191. Bertini E, D'Amico A, Gualandi F, Petrini S. Congenital muscular dystrophies: a brief review. Semin Pediatr Neurol 2011;18:277-88.
192. Ehrhardt J, Morgan J. Regenerative capacity of skeletal muscle. Curr Opin Neurol 2005;18:548-53.
193. Wagers AJ, Conboy IM. Cellular and molecular signatures of muscle regeneration: current concepts and controversies in adult myogenesis. Cell 2005;122:659-67.
194. Chachques JC, Shafy A, Duarte F, et al. From dynamic to cellular cardiomyoplasty. J Card Surg 2002;17:194-200.
195. von Harsdorf R, Poole-Wilson PA, Dietz R. Regenerative capacity of the myocardium: implications for treatment of heart failure. Lancet 2004;363:1306-13.
196. Zvaritch E, Kraeva N, Bombardier E, et al. Ca2+ dysregulation in Ryr1(I4895T/wt) mice causes congenital myopathy with progressive formation of minicores, cores, and nemaline rods. Proc Natl Acad Sci U S A 2009;106:21813-8.
197. Dowling JJ, Arbogast S, Hur J, et al. Oxidative stress and successful antioxidant treatment in models of RYR1-related myopathy. Brain 2012;135:1115-27.
198. Campbell KP, Stull JT. Skeletal muscle basement membrane-sarcolemma-cytoskeleton interaction minireview series. J Biol Chem 2003;278:12599-600.
199. Badylak SF. The extracellular matrix as a scaffold for tissue reconstruction. Semin Cell Dev Biol 2002;13:377-83.
200. Huijing PA, Jaspers RT. Adaptation of muscle size and myofascial force transmission: a review and some new experimental results. Scand J Med Sci Sports 2005;15:349-80.
201. Karpati G. Disorders of voluntary muscle. 8th ed. Cambridge ; New York: Cambridge University Press; 2010.
202. Sanes JR. The basement membrane/basal lamina of skeletal muscle. J Biol Chem 2003;29:29.
203. Le Grand F, Rudnicki MA. Skeletal muscle satellite cells and adult myogenesis. Curr Opin Cell Biol 2007;19:628-33.
204. Yurchenco PD, Patton BL. Developmental and pathogenic mechanisms of basement membrane assembly. Curr Pharm Des 2009;15:1277-94.
205. Mayer U. Integrins: redundant or important players in skeletal muscle? J Biol Chem 2003;278:14587-90.
206. Michele DE, Campbell KP. Dystrophin-glycoprotein complex: post-translational processing and dystroglycan function. J Biol Chem 2003;278:15457-60. Epub 2003 Jan 29.
207. Rutkowski A, Bonnemann C, Brown S, et al. Report on the Myomatrix Conference April 22-24, 2012, University of Nevada, Reno, Nevada, USA. Neuromuscul Disord 2012.
208. Hessle H, Engvall E. Type VI collagen. Studies on its localization, structure, and biosynthetic form with monoclonal antibodies. J Biol Chem 1984;259:3955-61.
209. Keene DR, Engvall E, Glanville RW. Ultrastructure of type VI collagen in human skin and cartilage suggests an anchoring function for this filamentous network. J Cell Biol 1988;107:1995-2006.
210. Ritty TM, Roth R, Heuser JE. Tendon cell array isolation reveals a previously unknown fibrillin-2-containing macromolecular assembly. Structure (Camb) 2003;11:1179-88.
211. Beighton P, De Paepe A, Steinmann B, Tsipouras P, Wenstrup RJ. Ehlers-Danlos syndromes: revised nosology, Villefranche, 1997. Ehlers-Danlos National Foundation (USA) and Ehlers-Danlos Support Group (UK). Am J Med Genet 1998;77:31-7.
212. Bosman FT, Stamenkovic I. Functional structure and composition of the extracellular matrix. J Pathol 2003;200:423-8.

213. Baumann M, Giunta C, Krabichler B, et al. Mutations in FKBP14 cause a variant of Ehlers-Danlos syndrome with progressive kyphoscoliosis, myopathy, and hearing loss. Am J Hum Genet 2012;90:201-16.
214. Kitamura M, Shimizu M, Ino H, et al. Collagen remodeling and cardiac dysfunction in patients with hypertrophic cardiomyopathy: the significance of type III and VI collagens. Clin Cardiol 2001;24:325-9.
215. Alexopoulos LG, Youn I, Bonaldo P, Guilak F. Developmental and osteoarthritic changes in Col6a1-knockout mice: biomechanics of type VI collagen in the cartilage pericellular matrix. Arthritis Rheum 2009;60:771-9.
216. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992;69:11-25.
217. Yao CC, Ziober BL, Squillace RM, Kramer RH. Alpha7 integrin mediates cell adhesion and migration on specific laminin isoforms. J Biol Chem 1996;271:25598-603.
218. Collo G, Starr L, Quaranta V. A new isoform of the laminin receptor integrin alpha 7 beta 1 is developmentally regulated in skeletal muscle. Journal of Biological Chemistry 1993;268:19019-24.
219. Song WK, Wang W, Sato H, Bielser DA, Kaufman SJ. Expression of alpha 7 integrin cytoplasmic domains during skeletal muscle development: alternate forms, conformational change, and homologies with serine/threonine kinases and tyrosine phosphatases. Journal of Cell Science 1993;106:1139-52.
220. Ziober BL, Vu MP, Waleh N, Crawford J, Lin CS, Kramer RH. Alternative extracellular and cytoplasmic domains of the integrin alpha 7 subunit are differentially expressed during development. Journal of Biological Chemistry 1993;268:26773-83.
221. Hodges BL, Hayashi YK, Nonaka I, Wang W, Arahata K, Kaufman SJ. Altered expression of the alpha7beta1 integrin in human and murine muscular dystrophies. J Cell Sci 1997;110 (Pt 22):2873-81.
222. Bao ZZ, Lakonishok M, Kaufman S, Horwitz AF. Alpha 7 beta 1 integrin is a component of the myotendinous junction on skeletal muscle. Journal of Cell Science 1993;106:579-89.
223. Echtermeyer F, Schober S, Poschl E, von der Mark H, von der Mark K. Specific induction of cell motility on laminin by alpha 7 integrin. J Biol Chem 1996;271:2071-5.
224. Schuler F, Sorokin LM. Expression of laminin isoforms in mouse myogenic cells in vitro and in vivo. J Cell Sci 1995;108 (Pt 12):3795-805.
225. Yao CC, Breuss J, Pytela R, Kramer RH. Functional expression of the alpha 7 integrin receptor in differentiated smooth muscle cells. J Cell Sci 1997;110 (Pt 13):1477-87.
226. Hodges BL, Hayashi YK, Nonaka I, Wang W, Arahata K, Kaufman SJ. Altered expression of the alpha7beta1 integrin in human and murine muscular dystrophies. Journal of Cell Science 1997;110:2873-81.
227. Belkin AM, Zhidkova NI, Balzac F, et al. Beta 1D integrin displaces the beta 1A isoform in striated muscles: localization at junctional structures and signaling potential in nonmuscle cells. J Cell Biol 1996;132:211-26.
228. van der Flier A, Kuikman I, Baudoin C, van der Neut R, Sonnenberg A. A novel beta 1 integrin isoform produced by alternative splicing: unique expression in cardiac and skeletal muscle. FEBS Lett 1995;369:340-4.
229. Zhidkova NI, Belkin AM, Mayne R. Novel isoform of beta 1 integrin expressed in skeletal and cardiac muscle. Biochem Biophys Res Commun 1995;214:279-85.
230. Cohn RD, Mayer U, Saher G, et al. Secondary reduction of alpha7B integrin in laminin alpha2 deficient congenital muscular dystrophy supports an additional transmembrane link in skeletal muscle. J Neurol Sci 1999;163:140-52.
231. Rooney JE, Welser JV, Dechert MA, Flintoff-Dye NL, Kaufman SJ, Burkin DJ. Severe muscular dystrophy in mice that lack dystrophin and alpha7 integrin. J Cell Sci 2006;119:2185-95.
232. Suzuki A, Yoshida M, Hayashi K, Mizuno Y, Hagiwara Y, Ozawa E. Molecular organization at the glycoprotein-complex-binding site of dystrophin. Three dystrophin-associated proteins bind directly to the carboxy-terminal portion of dystrophin. Eur J Biochem 1994;220:283-92.
233. Vachon PH, Xu H, Liu L, et al. Integrins (alpha7beta1) in muscle function and survival. Disrupted expression in merosin-deficient congenital muscular dystrophy. J Clin Invest 1997;100:1870-81.
234. Doe JA, Wuebbles RD, Allred ET, Rooney JE, Elorza M, Burkin DJ. Transgenic overexpression of the alpha7 integrin reduces muscle pathology and improves viability in the dy(W) mouse model of merosin-deficient congenital muscular dystrophy type 1A. J Cell Sci 2011;124:2287-97.

235. Hayashi YK, Chou FL, Engvall E, et al. Mutations in the integrin alpha7 gene cause congenital myopathy. Nat Genet 1998;19:94-7.
236. Nakashima H, Kibe T, Yokochi K. 'Congenital muscular dystrophy caused by integrin alpha7 deficiency'. Dev Med Child Neurol 2009;51:245.
237. Mayer U, Saher G, Fässler R, et al. Absence of integrin α7 causes a novel form of muscular dystrophy. Nature Genetics 1997;17:318-23.
238. Pegoraro E, Cepollaro F, Prandini P, et al. Integrin alpha 7 beta 1 in muscular dystrophy/myopathy of unknown etiology. Am J Pathol 2002;160:2135-43.
239. Aumailley M, Specks U, Timpl R. Cell adhesion to type VI collagen. Biochemical Society Transactions 1991;19:843-7.
240. Pfaff M, Aumailley M, Specks U, Knolle J, Zerwes HG, Timpl R. Integrin and Arg-Gly-Asp dependence of cell adhesion to the native and unfolded triple helix of collagen type VI. Experimental cell research 1993;206:167-76.
241. Dubowitz V, Sewry CA, eds. Muscle biopsy: A practical approach. 3rd ed. London: Elsevier; 2006.
242. Ellis JD, Barrios-Rodiles M, Colak R, et al. Tissue-specific alternative splicing remodels protein-protein interaction networks. Mol Cell 2012;46:884-92.
243. Pan Q, Shai O, Misquitta C, et al. Revealing global regulatory features of mammalian alternative splicing using a quantitative microarray platform. Mol Cell 2004;16:929-41.
244. Wang ET, Sandberg R, Luo S, et al. Alternative isoform regulation in human tissue transcriptomes. Nature 2008;456:470-6.
245. Royce PM, Steinmann BU. Connective tissue and its heritable disorders : molecular, genetic, and medical aspects. New York: Wiley-Liss; 1993.
246. Higuchi I, Shiraishi T, Hashiguchi T, et al. Frameshift mutation in the collagen VI gene causes Ullrich's disease. Ann Neurol 2001;50:261-5.
247. Yao CC, Ziober BL, Squillace RM, Kramer RH. Alpha7 integrin mediates cell adhesion and migration on specific laminin isoforms. Journal of Biological Chemistry 1996;271:25598-603.
248. Wiberg C, Heinegard D, Wenglen C, Timpl R, Morgelin M. Biglycan Organizes Collagen VI into Hexagonal-like Networks Resembling Tissue Structures. J Biol Chem 2002;277:49120-6.
249. Bowe MA, Mendis DB, Fallon JR. The small leucine-rich repeat proteoglycan biglycan binds to alpha- dystroglycan and is upregulated in dystrophic muscle. J Cell Biol 2000;148:801-10.
250. Rafii MS, Hagiwara H, Mercado ML, et al. Biglycan binds to alpha- and gamma-sarcoglycan and regulates their expression during development. J Cell Physiol 2006;209:439-47.
251. Young MF, Fallon JR. Biglycan: a promising new therapeutic for neuromuscular and musculoskeletal diseases. Curr Opin Genet Dev 2012.
252. Useful Information on Rare Diseases from an EU Perspective. In: General ECHaCPD-, ed. Commission Europeenne. Luxembourg; 2009.
253. Congress t. Rare Disease Act of 2002. In: Government US, ed. Washington, D.C.: U.S. Government Printing Office; 2002:116.
254. Scott E, Eagle M, Mayhew A, et al. Development of a functional assessment scale for ambulatory boys with Duchenne muscular dystrophy. Physiother Res Int 2012;17:101-9.
255. Mayhew A, Cano S, Scott E, Eagle M, Bushby K, Muntoni F. Moving towards meaningful measurement: Rasch analysis of the North Star Ambulatory Assessment in Duchenne muscular dystrophy. Dev Med Child Neurol 2011;53:535-42.
256. Bonnemann CG, Rutkowski A, Mercuri E, Muntoni F. 173rd ENMC International Workshop: congenital muscular dystrophy outcome measures 5-7 March 2010, Naarden, The Netherlands. Neuromuscul Disord 2011;21:513-22.
257. Steffensen B, Hyde S, Lyager S, Mattsson E. Validity of the EK scale: a functional assessment of non-ambulatory individuals with Duchenne muscular dystrophy or spinal muscular atrophy. Physiother Res Int 2001;6:119-34.
258. Collins J, Bonnemann CG. Congenital muscular dystrophies: toward molecular therapeutic interventions. Curr Neurol Neurosci Rep 2010;10:83-91.
259. Erb M, Meinen S, Barzaghi P, et al. Omigapil ameliorates the pathology of muscle dystrophy caused by laminin-alpha2 deficiency. J Pharmacol Exp Ther 2009;331:787-95.
260. De Bari C, Dell'Accio F, Vandenabeele F, Vermeesch JR, Raymackers JM, Luyten FP. Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. J Cell Biol 2003;160:909-18.
261. Meng J, Adkin CF, Arechavala-Gomeza V, Boldrin L, Muntoni F, Morgan JE. The contribution of human synovial stem cells to skeletal muscle regeneration. Neuromuscul Disord 2010;20:6-15.

262. Olanow CW, Schapira AH, LeWitt PA, et al. TCH346 as a neuroprotective drug in Parkinson's disease: a double-blind, randomised, controlled trial. Lancet Neurol 2006;5:1013-20.
263. Miller R, Bradley W, Cudkowicz M, et al. Phase II/III randomized trial of TCH346 in patients with ALS. Neurology 2007;69:776-84.