Genetic heterogeneity in collagen VI-related myopathy

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Abstract

Since discovery of *COL6A1*, *A2* and *A3* as disease-causing genes in collagen VI-related myopathies, several investigations have been done to identify novel genes which are responsible for collagen VI-related myopathy phenotypes with no mutations in *COL6A1-A3* genes. By exome sequencing of five affected individuals from pedigrees lying in the phenotypic spectrum of Bethlem-like myopathy but no mutations in *COL6A1-A3* genes, we identified two missense and one splice variant mutation acting as dominant negative in the gene *COL12A1* in three pedigrees with overall six affected individuals. *In silico* analysis predicted pathogenecity of these mutations. Immunohistochemical studies revealed in two missense mutations misfolded collagen XII could secrete to extracellular matrix but could not assemble to its structure, while for the splice variant mutation the misfolded protein could not secrete out of the cells. Phenotypic spectrum of collagen XII affected individuals from our cohort included mild to moderate progressive muscle weakness, joint hyperlaxity, skin involvements such as keloid formation and scoliosis.

Studying suprastructure of extracellular matrix via electron microscopy in the affected individuals by *in vitro* collagen fibrillogenesis of concentrated collagen extracted from cell culture and collagen fibrils surrounded cultured fibroblast, we could not reveal any differences in fibril diameter. Adult fibroblast/ C2C12 coculture demonstrated extracellular matrix of affected individuals did not have properties which are required for increasing myoblast differentiation as it was seen in intact extracellular matrix.

Our investigation on Bethlem-like myopathy heterogeneity introduced *COL12A1* as the new disease-causing gene in Bethlem-like phenotype with no mutations in *COL6A1-A3* genes. However, more research in this field is required to decipher pathogenic mechanism of collagen XII mutation.

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Declarations

I, GolaraTorabiFarsani, declare that the presented thesis is the result of my own original research. Any type of other works which is indicated in my thesis is properly referenced and acknowledged. I declare that none of these works has been submitted for other academic degrees. Part of this research which has been submitted for prior publication is properly referenced.

Selected Publications

Salih, M.A., Seidhamed, M.Z., Elkhashb H., Hicks, D.Laval, S.,**Farsani, G.T**, Muller, J. Lochmuller, H. Salbutamol benefits children with congenital myasthenic syndrome due to ALG2 mutation. October 2015, DOI: 10.1016/j.jns.2015.08.261

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List of Abbreviations

AD Autosomal Dominant

ALG2 gene Asparagine link glycosylation2 gene

AR Autosomal ressecive

BM Bethlem myopathy

Buffer EB Ellution Buffer, QIAGENE company protocol

Buffer PB Binding Buffer, QIAGENE company protocol

Buffer PE Wash Buffer, QIAGENE company protocol

Buffer QG Wash and Solubilize Buffer, QIAGENE company protocol

Bp Base pair

C Cysteine

Cat no Catalogue number

˚C Degrees Celsius

CCD Central Core

CGH Comparative Genomic Hybridization

CMD Congenital Muscular Dystrophy

CsA Cyclosporine A

CK Creatine Kinase

Col Collagen

Col VIA Collagen 6A

Col XIIA Collagen 12A

cDNAComplementry Deoxyribonuvleic acid

D Aspartic acid

DAPI4',6-diamidino-2-phenylindole

DEPC water Diethyl pyrocarbonate water

DGC Dystrophin-Glycoprotein Complex

DMEMDulbeco’s Modified Eagle Media

DNA Deoxyribonucleic acid

dATPsDeosxyadenosine triphosphate

dCTPsDeoxycytidine triphosphate

dGTPsDeoxyguanosine triphosphate

dNTPsDeoxyribonucleotide triphosphate

dTTPsDeoxytimidine triphosphate

deldeletion

ECM ExtraCellular Matrix

EDMD Emery-Dreifuss Muscular Dystrophy

EDS Ehlers-Danlos Syndrome

EMG Electromyography

Fwd Forward Primer

FACIT Fibril Associated Collagen Interrupted Triples

FBN3 gene Fibrilline 3 gene

FKRP gene Fukutin related protein gene

G Glycine

IF Immunofluorescence

Indel Insertion/deletion

Ins Insertion

L Leucine

LGMD Limb Girdle Muscular Dystrophy

M Molar (1 mol/L)

m mol

ml millilitre

mM millimolar

MDC1A Merosin-Defi cient Congenital Muscular Dystrophy Type 1A

MDC1C Congenital Muscular Dystrophy Type 1C

M DC1D Congenital Muscular Dystrophy Type 1D

MRI Magnetic Resonance Imaging

mRNA Messenger Ribonucleic Acid

NA Not Applicable

NHDFCs Normal Dermal Human Fibroblasts Cells

NCBI National Centre for Biotechnology

Ng Nanogram

PBS Phosphate Buffered Saline

Tm Melting Temperature

PCR Polymerase chain reaction

Pmol Picomole

PTP Permeability Transition Pore

Rev Revers Primer

R Arginine

RNA Ribonucleic acid

SNP: Single Nucleotide Polymorphism

S: Serine

siRNAShort Interfering Ribonucleic Acid

SR Sarcoplasmic Reticulum

T Temperature

TGF-β Transforming Growth Factor beta

U/µl Units/microliter

UCMD Ullrich Congenital Muscular Dystrophy

UCSC Genome Browser The human genome browser at the University of California, Santa Cruz

vWFA von Willebrand Factor Type A

W Tryptophan

M Microgram

MlMicrolitre

µMMicromol

Chapter 1 Introduction

1.1 Neuromuscular disorders classification and description:

Neuromuscular disorders (NMD) are described as progressive muscle weakness which could lead to pronounced physical disabilities (de Visser and Oliver, 2017).These disorders encompass a spectrum of diseases where peripheral nervous system from the anterior horn cell which is part of spinal cord that enervate axial body muscles, nerve fibres, the neuromuscular junction to the muscle can be involved (Turakhia *et al.*, 2013). All types of neuromuscular disorders share the unifying aspect of abnormal muscle function. This pathologic function could result in abnormalities and disease symptoms (Birnkrant and Noritz, 2008). Because of the progressive nature of neuromuscular disorders, the symptoms also include chronic symptoms such as impaired to loss of ambulation, joint contractures or joint hyperlaxities, skeletal abnormalities (particularly scoliosis), cardiac abnormalities and respiratory failure. Signs of dynamic impairments such as fatigable weakness, myalgia, rhabdomyolysis and exercise intolerance are also present in neuromuscular disorders (Turakhia *et al.*, 2013).

Paediatric neuromuscular disorders represent a sub-group of neuromuscular disorders with onset in childhood (Al-Ghamdi *et al.*, 2017). Most paediatric neuromuscular disorders have genetic etiology. Like other genetic disorders, these diseases lead to lifelong morbidities and often childhood mortality. In addition, possible and potential treatments for these disorders are rare and yet premature (Dowling *et al.*, 2017).

Paediatric neuromuscular disorders with genetic basis can be presented as de novo – Genetic variant that arise in children but are not present in either parents – or inherited disorders. For those with known and underpinning genetic etiology, the pattern of inheritance can be autosomal dominant or autosomal recessive or X-linked. While among all pediatric NMDs the untreatable ones are prevailing the others, identifying the genetic underlying cause for these disorders become an important issue. Recognizing etiologic basis of these disorders helps to find right palliative care and genetic consultation. Neuromuscular disorders are caused by wide variety of genes. So far over three hundred different genes were identified which associated with different neuromuscular disorders (Dohrn *et al.*, 2017). In this section, we describe the different and most common pediatric neuromuscular disorders with genetic basis.

1.1.1 *Muscular Dystrophies (MD):*

Muscular Dystrophies are a clinically and genetically variable group of disorders unified by presence of muscle weakness and its following disabilities and a dystrophic pattern observed in muscle in biopsies. Generally, muscular dystrophies can be categorized as: dystrophinopathies (DMD and Becker MD), myotonic dystrophies, limb girdle muscular dystrophies, Emery-Dreifus muscular dystrophies and congenital muscular dystrophies. There are common themes in diagnostic and treatment approaches related to all these muscular dystrophies (Dowling *et al.*, 2017).

Duchenne muscular dystrophy (DMD) is one of the most common pediatric neuromuscular conditions. It presents during infancy by progressive muscle weakness. However, other organs such as heart, brain and smooth muscles will involve. Dilated cardiomyopathy and respiratory failure are two common symptoms. This X-linked recessive primary muscle disorder has a prevalence of 1 in 5000 boys. It is mostly caused by out of frame deletion in the gene DMD, which encodes Dystrophin, a membrane stabilizer protein. In 1956 Dubowitz described a combination of splints, physiotherapy and antibiotics as treatment for this disorder (Dowling *et al.*, 2017). Since then, with help of multi-disciplinary treatments DMD is not a childhood life-limiting disorder. Furthermore, continuous efforts are undergoing to develop mutation-specific treatment approaches for this disorder (Aartsma-Rus *et al.*, 2017b). Becker muscular dystrophy is another dystrophinopathy with milder symptoms compared to DMD. Typically BMD starts after first decade of life, loss of ambulation occurred in the third decade of life and cardiomyopathy is variable. Typically, BMD patients have longer life expectancy compared to DMD. Becker muscular dystrophy is result of a small, partially functional protein due to a deletion in genetic code which does not lead to frame shift variation (Dohrn *et al.*, 2017).

Myotonic Dystrophy (MD) is a systemic disorder caused by CTG repeats expansion that results in accumulation of toxic mRNA in the nucleus. MD can be categorized in two main types: Type 1 which presents in childhood or early adulthood with weakness of facial muscles and distal extremities. In MD1 the triplet expansions (50 to>3500) occur in 3` UTR of the Dystrophia Myotonica protein kinase (*DMPK)* gene locus. MD type 2 is due to CCTG expansion (75 to approximately 1100) in the first intron of the gene Zinc finger protein 9 (*ZFN9*). At birth, hypotonia, respiratory problems and feeding difficulties are cardinal features. During infancy these patients develop cardiac problems, delayed motor

milestones and learning disabilities including features of autism spectrum. Some non-muscle related symptoms such as cardiac arrhythmia, childhood cataract, dysphagia, mental retardation and infertility with insulin-resistance are present in Myotonic Dystrophies. At the moment, most non-genetic based therapies aim to alleviate non-neuromuscular symptoms. Genetic approaches to reduce the amount of toxic RNA are under investigation (Dowling *et al.*, 2017).

Limb Girdle Muscular Dystrophies (LGMDs) are a group of genetically diverse and clinically heterogeneous muscular dystrophies. LGMDs symptoms usually start after the first year of life with limb girdle progressive muscular weakness, dystrophic muscle biopsies and high Creatine kinase (CK) level. LGMDs are subclassified into two major groups: Autosomal dominant LGMD or LGMD1 and autosomal recessive LGMD or LGMD2. So far, more than eight genes are associated with LGMD1 (LGMD1A-LGMD1H) and at least twenty three genes lead to LGMD2 (LGMD2A-LGMD2W). In total, over 50 genetic loci were identified in LGMDs. The diverse heterogeneity highlights the problem of clinical diagnosis difficulties and various differential diagnosis (Monies *et al.*, 2016). Autosomal dominant form of LGMD is less common and responsible for 10-15% of the disease frequency. Overall frequency of LGMDs is between14000 to 45000. Among different types of LGMDs, type 2A, also known as primary calpainopathy due to mutations in *CAPN3* at 25-30% is the most common form of LGMD. LGMD2I accounts for 20% of LGMD cases and caused by mutations in *FKRP* gene .Mutations in other genes are rare in LGMDs and usually cause adult-onset LGMDs like dysferlinopathy and *ANO5* related LGMD. Several pharmaceutical and genetical approaches are undergoing to treat LGMDs, while few investigations lead to clinical trials like AVV based gene therapy for LGMD2I and calpainopathy (Bushby *et al.*, 2007; Patel *et al.*, 2008).

Emery-Dreifuss Muscular Dystrophy (EDMD) is a muscular dystrophy caused by mutations in various genes. However, most EDMD cases are associated with Lamin A (*LMNA*) or *EMERIN* mutations. The hall mark of EDMDs clinical presentation is muscle weakness of the scapula-peroneal region with a range of severity. EDMD can be life-limiting in many cases like *LMNA* associated EDMD which demonstrated with rapid progression of muscle weakness, severe contractures and early death. There is a significant clinical and genetical overlap between EDMD and other neuromuscular or non-muscular diseases (such as lipodystrophy and progeria). Palliative treatments for some

symptoms of the disease, such as fatal cardiac arrhythmia is one of the key elements of therapeutic plan in these patients. Genetic-based treatments such as exon-skipping for exon 3 or 5 of *LMNA* gene, adenovirus therapies for *EMERIN* or allele specific-silencing are of consideration (Dowling *et al.*, 2017).

Congenital Muscular Dystrophies (CMDs) are a group of muscular dystrophies with common age of onset. Usually these diseases start twelve to eighteen months after birth. Symptoms include progressive muscle weakness, skeletal deformity, joint and skin involvement. CMDs according to genetic cause can be categorized in three different subgroups: *LAMA2*-related CMDs (merosin deficient CMDs or MDC1A), collagen VI-related (Ullrich CMD and Bethlem myopathy) and dystroglycaopathies. Like other muscular dystrophies, CMDs represent a spectrum of heterogenic and clinically diverse group of disorders. Other rare subtypes of CMDs include Marinesco-Sjogern syndrome, *LMNA*-related muscular dystrophies and rigid spine muscular dystrophies (Mercuri and Muntoni, 2012).

At present, palliative treatments for CMD symptoms are applied in patients. However, many genetic approaches included AAV deliveries and CRISPR/CAS 9 for MDCA1, allele specific antisense oligonucleotide for collagen VI-related muscular dystrophy and molecular approached to improve muscle-matrix adhesion in different CMDs has been tested (Bolduc *et al.*, 2014; Dowling *et al.*, 2017). Collagen VI-related muscular dystrophies, as the main focus of this research will be described in details later.

1.1.2 *Spinal Muscular atrophy (SMA):*

SMA is another common pediatric disorder caused by different genes. However, the most common genetic cause is mutations in *SMN1* gene. This is an autosomal recessive disorder with incidence of 1 in 6000 to 1 in 10000 live birth per year. Atrophy of myofibres under the microscope is the hallmark of the disease. However, a dramatic genetic and phenotypic variation was observed in SMAs with four types of childhood onset (type 0,I,II,III) and one type of adult onset (type IV).Type I SMA or Werdnig-Hoffman disease is the most common one with a deteriorating course and high death incidence. Clinical manifestations of SMA include hypotonia with poor head control, proximal muscle weakness, feeding and respiratory problems, weak cough and weak cry which start before six month of age. Severe forms can never sit. A centromeric gene to *SMN1* called *SMN2* act as the main modifier for SMA phenotypic variation. *SMN2* encodes a protein with 20%

activity of SMN1 protein. The increased copy number variant of the *SMN2* gene lead to a milder SMA phenotype (Aartsma-Rus *et al.*, 2017a). Gene therapies based on modification of SMN2 protein for therapeutic approaches are under investigation (Ramirez *et al.*, 2018). Moreover, Nusinersen, an antisense oligonucleotide which target *SMN2* exon 7 to include it in final transcript shows promising result in clinical trials (Scoto *et al.*, 2017). However, treatment approaches for this disorder are still under development and SMA is still one of the common cause of death during childhood (Dowling *et al.*, 2017).

1.1.3 *Charcot-Marie-Tooth disease (CMT):*

CMT is one of the most common paediatric neuropathies with a prevalence of 1 in 2500 births. A marked phenotypic and genetic variation is associated with Charcot-Marie-Tooth (CMT) disorder. To date, mutations in over 80 genes have been identified as disease-causing. However, the majority of cases are caused by mutations in four genes: *PMP22, MPZ*, *GJB32* and *MFN2*. Duplication in *PMP22*so far are the most common cause of CMT. Peripheral nerve fibres are the primary pathologic region in CMT disease. Also the disorder could involve axon or the myelination of Schwan cells. CMT is subcategorized depending on the mode of inheritance (dominant vs recessive) and location of pathology. CMT1, CM2 and CM3 caused by neuron demyelination, while CM4 is due to axonal demyelination. Progressive distal muscle weakness, hand and food deformities and loss of sensory perception are the common clinical symptoms and signs of CMT patients. Genetic based therapies focus on decreasing PMP22 level as this is the mutation of excessive protein dosage. Like other paediatric neuropathies the success of different treatment strategies in CMT is limited and under investigation (Dohrn *et al.*, 2017).

1.1.4 *Congenital myasthenic syndromes (CMS):*

CMSs are disorders caused by mutations that affect or dysregulate the neuromuscular junction. So far near 30genes have been identified as disease causing. Mutations in the acetylcholine receptor subunit epsilon (*CHRNE*) are the most frequent cause of CMS. Phenotypically, Congenital Myasthenic Syndrome is characterized by facial, mainly ocular and bulbar, and muscle weakness which has a fluctuating pattern. Ptosis is one common signs in CMS patients. Autoimmune myasthenia gravis has got common symptoms with CMS and is a differential diagnosis. Congenital myasthenic syndrome has got a defect-based classification, disease-onset, with postsynaptic mutation as the most common form (~70%) and presynaptic, synptic and synptopathic as other forms. Severity and response to treatment is markedly variable among CMS patients. Cholinesterase inhibitor,

Pyridostigmine, is the primary treatment in most patients. However, the response to drug is variable with some patients showing no effect or even worsening of symptoms. The treatment for slow channel CMS-a particular postsynaptic form of CMS with severe progression and loss of ambulation in adult stage-(due to gain of function mutation) could worsen the symptoms. Whether due to the rarity of the disease or relative effectiveness of present drugs, other therapeutic approaches for CMS patients` are not well developed. Given the fact that CMS patients response to drugs is variable depending on the genetic base of the disease, identifying genetic subtypes in these patients is very important (Chaouch *et al.*, 2012; Nicole *et al.*, 2014).

1.1.5*Congenital Myopathies (CMs):*

CMs are a group of largely variable neuromuscular disorders in terms of genetic cause. Clinical presentations of CMs include a spectrum of severe to non-progressive muscle weakness, hypotonia and respiratory involvement. Like congenital muscular dystrophies (CMDs), CMs start at infancy but could be distinguished from CMDs by non-dystrophic muscle appearance and normal serum CK levels. Core myopathies, Centronuclear myopathies, Nemaline rod myopathies and fibre type disproportion are the most common examples of congenital myopathies. So far, mutations in over 20 genes are associated with CMs and ryanodine receptor (*RYR1*) mutations are the most common cause. While no approved drugs exist for CM patients, several clinical trials for drugs such as Dantrolen for *RYR1* mutations and L-tyrosin for nemalin myopathy are undergoing research. AAV based therapies are also in consideration (Dowling *et al.*, 2017).

As mentioned above paediatric neuromuscular disorders are a group of large and heterogeneous disease. Clinical presentations of these disorders are markedly variable for one disease but overlapping between different groups of neuromuscular disorders. Considering this fact, treatment of these diseases is complicated. One major advance which could benefit prognosis, consultation and treatment of neuromuscular disorders is the identification of the underlying genetic cause (Dohrn *et al.*, 2017).

1.2 Collagen VI-related myopathies:

Collagen VI- related myopathies comprise a spectrum of diseases with Ullrich congenital muscular dystrophy (UCMD) in the severe end and Bethlem myopathy(BM) in the milder end of the spectrum (Bonnemann, 2011). Collagen VI-related myopathies like other neuromuscular disorders, are a group of heterogeneous and clinically diverse disorders.

COL6A1, *A2*, *A3* genes which encode heterotrimeric microfibrillar collagen VI protein were the first genes to be recognized in collagen VI-related myopathies as pathogenic (Lampe *et al.*, 1993). Collagen VI-related myopathies, are a subgroup of congenital muscular dystrophies (CMDs), this group of disorders are unified by progressive muscle weakness and dystrophic signs in muscle biopsy (Bushby *et al.*, 2014).

UCMD was initially reported by Otto Ullrich (1930) as “Atonic-Sclerotic muscular dystrophy”. He characterized muscle weakness and joint hypermobility together with evolving and significant joint contractures in UCMD patients (Bonnemann, 2011). Ullrich congenital muscular dystrophy remained as a severe muscular entity mostly reported in European and Japanese patients. However, case reports of UCMD from different parts of the world have expanded its geographical prevalence (Furukawa and Toyokura, 1977; Allamand *et al.*, 2010; Bozorgmehr *et al.*, 2013).

Bethlem myopathy was initially reported by Bethlem and Van Vijngaarden (1976) in the Netherland. They recognized the disease as an autosomal-dominant disorder with progressive muscle weakness and joint hypermobility. Since then, several reports of Bethlem patients have been published. In 1988 the author of a report of Bethlem myopathy in a pedigree of French-Canadian individuals with four affected members suggested the name Bethlem myopathy for this distinct clinico-genetic entity (Mohire *et al.*, 1988).

Mutations in *COL6A* genes were first recognized in Bethlem myopathy as disease-causing in the pedigrees of large families from Netherland in 1996 (Jobsis *et al.*, 1996) and USA in 1998 (Pan *et al.*, 1998). It was after this discovery that scientists in Italy (Bertini and Peppe) noticed common clinical symptoms between Bethlem and Ullrich myopathies. This leadto the identification of collagen VI as disease-causing gene in Ullrich in 2001(Camacho Vanegas *et al.*, 2001). The term collagen VI-related myopathy was coined afterwards.

1.2.1 *Clinical presentation:*

As mentioned above Ullrich congenital muscular dystrophy and Bethlem myopathy are two distinct clinical entities which form the two ends of collagen VI related myopathy spectrum. However, intermediate phenotypes with overlapping features of UCMD and BM can be found in between.

1.2.1.1 *Ullrich congenital muscular dystrophy:*

Congenital muscular dystrophy type Ullrich (UCMD, MIM: 254090) presents at birth or the first year of life. However, like other neuromuscular disorders the diagnosis of UCMD cannot be made immediately after birth. UCMD patients present with hypotonia, muscle weakness and joint hyperlaxity. Distal joints can be extremely hyperlax allowing fingers to bend back to the dorsal surface of wrist or drop down to the wrist. This feature may mimic other neuromuscular disorders such as Ehlers Danlos syndrome. Joint hyperlaxity leads to congenital hip dislocation in about 50% of patients (Bonnemann, 2011). While distal joint hyperlaxity is a prominent feature in UCMD patients, some patients also present with proximal joint contractures at birth. Contractures are normally seen in hips, knees and elbows (Lampe *et al.*, 2008). Other signs of UCMD at birth includes torticollis (Spasm in neck muscle which leads to lateral bending of neck from central body axis), skeletal deformities such as kyphosis (internal bending of spine), kyphoscoliosis (internal and latheral bending of spine) and pes adductus (internal bending of foot from central body axis) Prominent calcaneus is another common sign. Overall 50% of UCMD patients shows skeletal system deformities (Bonnemann, 2011).

Presence of one of the above-mentioned signs in conjunction with delayed motor milestones suggests UCMD diagnosis. Connective tissue symptoms are a key factor in UCMD diagnosis and could differentiate between UCMD and other neuromuscular disorders.

Delayed motor milestones are progressive in UCMD. While most severe UCMD patients never achieve independent walking, some manage to crawl. Walking ability in others is diminished during childhood as a result of progressive muscle weakness or development of contractures in lower limb joints. At the median age of ten years old, most UCMD patients are wheelchair dependent. Muscle weakness affects both upper and lower limbs with preserved antigravity force in even severe patients (Bonnemann *et al.*, 2011).

Skin involvement is another symptom in UCMD. Excessive keloid formation after surgery is one example of skin signs. Hyperkeratosis pilaris (pathches of extra skin formation sometimes in different colour from normal skin) in extensor surfaces of upper limb is seen in these patients. The skin signs are not specific to UCMD patients. However, should be considered in most severe cases (Bonnemann *et al.*, 2011). Figure 1.1 and 1.2 shows

some signs and symptoms of collagen VI-related myopathies and particularly UCMD patients.

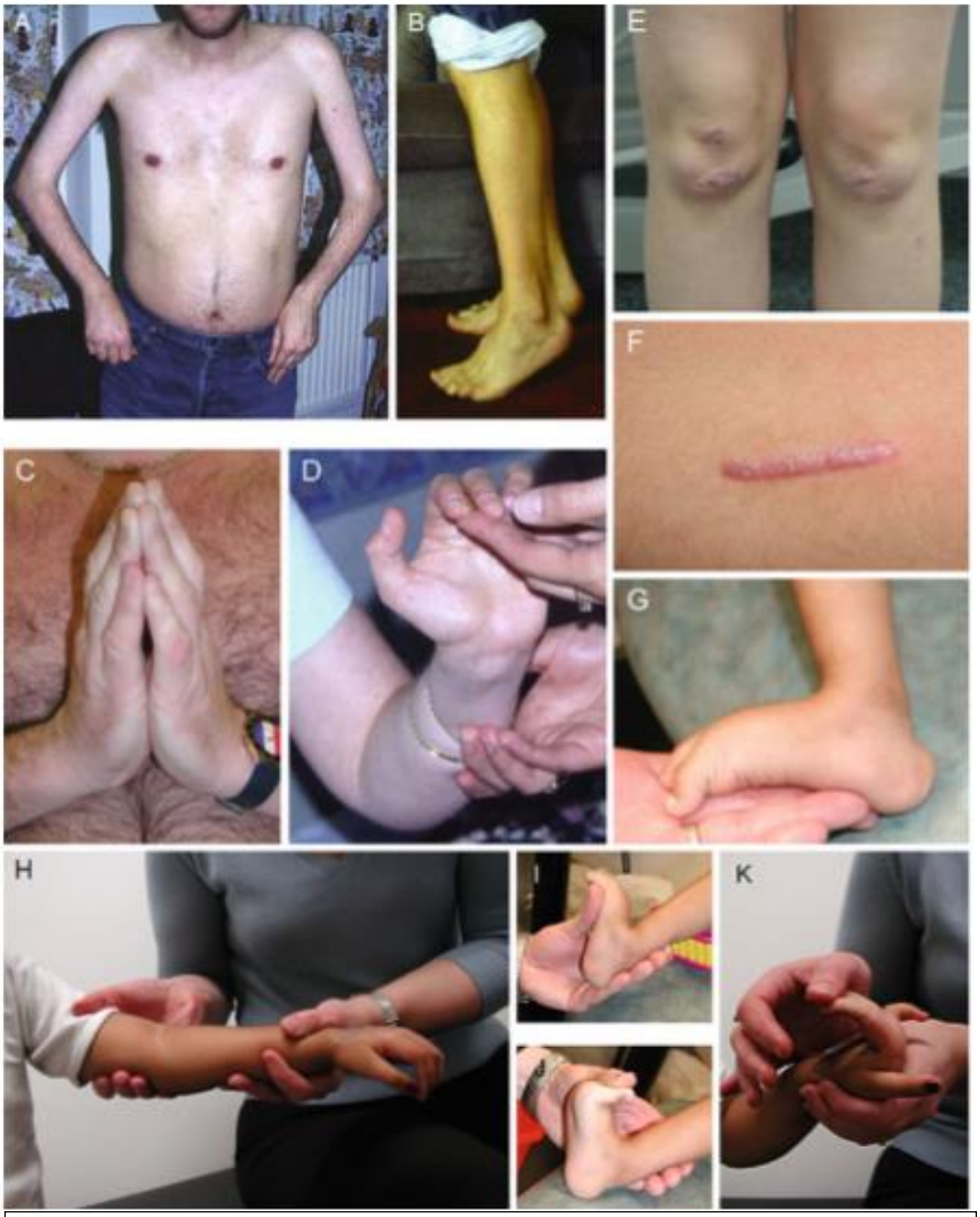


Figure 1.1.Different symptoms of collagen VI-related muscle patients. Contractures in elbow (A), ankle and knee (B), fingers(C,D), skin involvement such as hyperthrophy (E), kloid formation (F), joint contractures in foot fingers and hand fingers (G,H), joint hyperlaxities (I, J, K) (Bushby *et al.*, 2014).

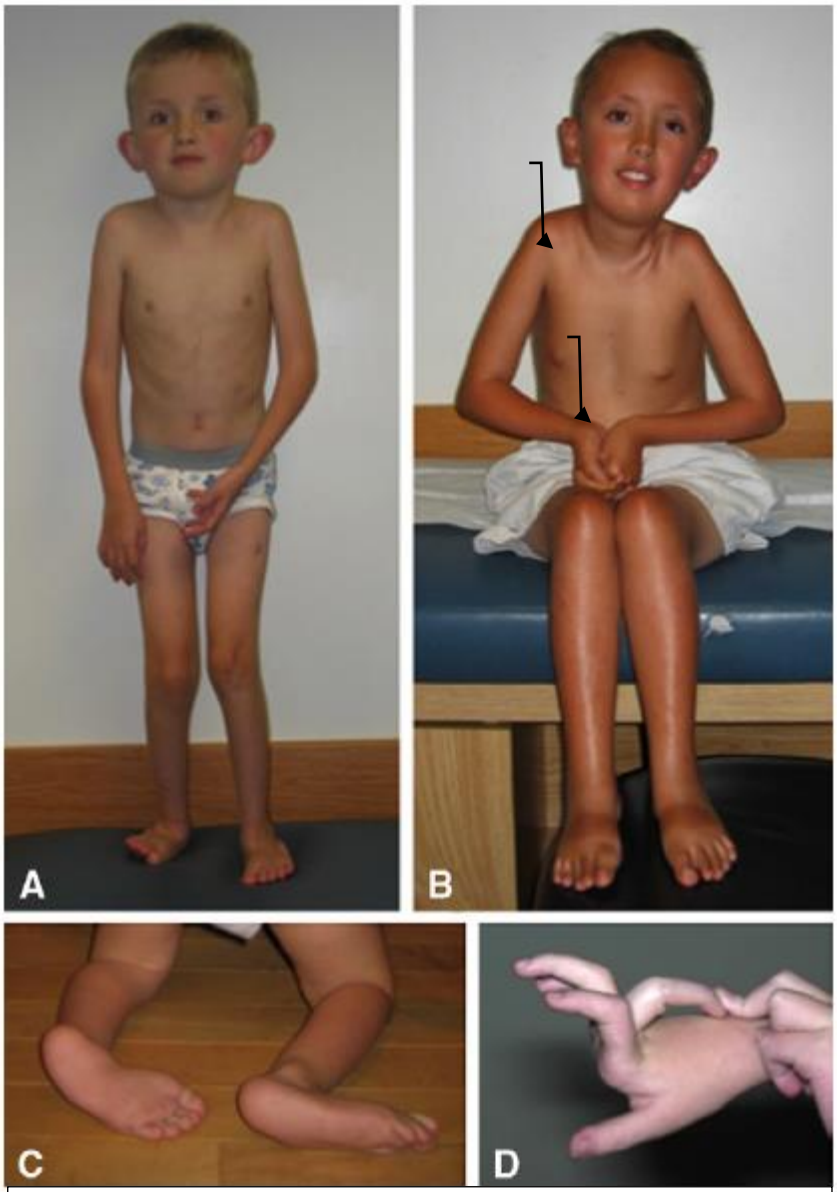


Figure 1.2.Clinical presentations of UCMD patients.(A) Patient able to walk with mild knee and elbow contracture. (B) Typical contractures of UCMD in elbow, pectoralis major, hips, knees. (C) Prominent calcaneus and striking hiperlaxity in toes finger. (D) Phalangeal hyperlaxity (Bonnemann, 2011).

Some other features of UCMD are respiratory involvement and feeding difficulties. Both symptoms occur in the context of muscle weakness. Restrictive pattern of pulmonary disease due to chest wall and diaphragmatic muscle weakness needs to be monitored in

these patients. Supportive care should be considered in most severe cases who develop esophagogastreal reflux or need to be fed through the stomach. Cardiac involvement is not reported for UCMD patients and intact cardiac system could help in differential diagnosis in these patients (Bushby *et al.*, 2014).

1.2.1.2 *Bethlem myopathy*:

Bethlem myopathy is another collagen VI-related myopathy disorder which form the milder end of collagen VI-related myopathy spectrum. Bethlem myopathy (BM, MIM:158810) could present at birth with symptoms such as torticoli- twisted neck or abnormal flexion of head or neck muscles-congenital deformity in which the heel is flexed inward and the foot is plantar flexed- and skeletal deformities such as equinovarus deformity (Skletal deformity with outward heel flexion) and foot dorsiflexor contractures (50%). Muscle weakness is either not present at birth or very mild. Contractures which present at birth will resolve during the first year of life. However, usually new contractures set in during the first decade of life. Contractures involve distal joints like Achilles tendon, elbows, pectoralis muscles, long finger flexors and particularly interphalangeal muscle of 2 to 5 digits. Adult affected individuals often cannot put the plantar surface of their hands together (Bonnemann, 2011). Figure 1.3 shows some signs of Bethlem myopathy.

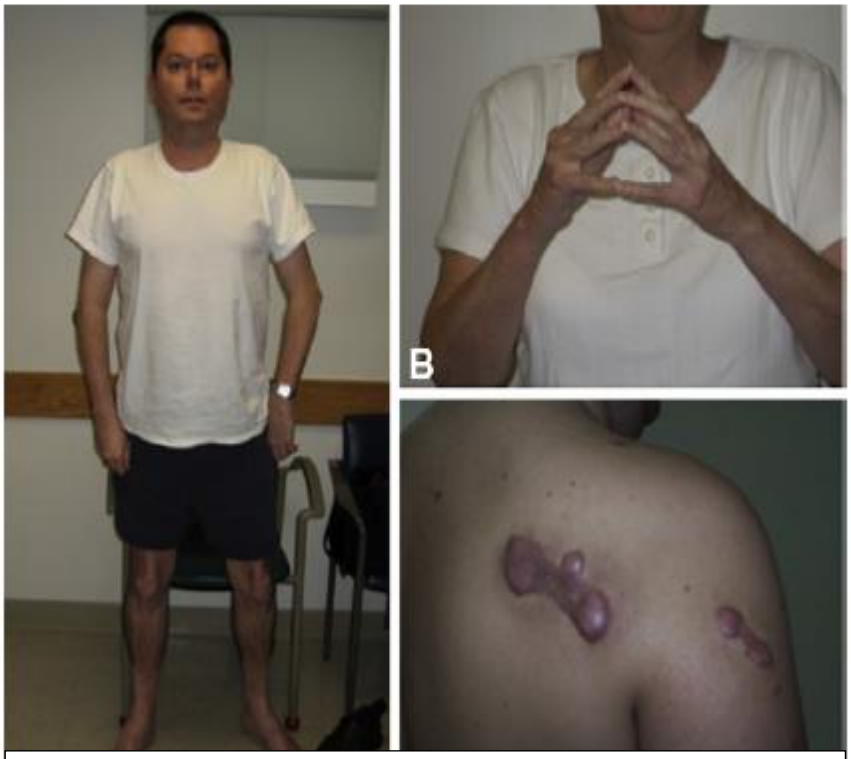


Figure 1.3.Clinical presentations of Bethlem myopathy. (A) Patients are able to stand and walk with elbow and knee contractures. (B) Typical finger contractures in Bethlem myopathy. (C) keloid formation in Bethlem myopathy (Bonnemann, 2011).

These contractures often progress during the course of the disease. Limited hand function due to contractures has been reported in BM patients. Distal joints hyperlaxity is another connective tissue sign in Bethlem patients. However, it might not be severe as Ullrich affected individuals.

Delayed motor milestones such as delay in ambulatory state or difficulties to run have been reported in BM patients. However, the weakness is less and has a stable pattern before the third decade of life. At this time, muscle weakness which is mostly present in proximal muscle starts to progress. By the age of fifty, around two thirds of BM patients lose their ambulation. However, many BM patients present a mild muscle weakness which they do not notice it themselves and it is only identified during neural examinations (Bonnemann *et al.*, 2011). Respiratory involvement is another feature of BM (Quijano-Roy *et al.*, 2014). Like UCMD patients, respiratory involvement results from rib cage muscle stiffness and weak diaphragmatic muscles. Respiratory symptoms are often seen in most

severe BM cases and monitoring is needed (Foley *et al.*, 2013). Skin involvement in BM patients is mostly present as keloid formation without velvety skin (Bushby *et al.*, 2014). Cardiac problems have been reported just in one case of BM with asymmetrical septal hypertrophy. This finding may represent an incidental sign (Bonnemann, 2011).

Signs and symptoms mentioned above are representing more classical features of Bethlem myopathy. However, a wide number of signs and symptoms are attributed to BM. Those with prominent proximal weakness and less joint involvements mimic limb girdle muscular dystrophy (LGMD), whereas those with less muscle weakness signs and more contractures could overlap with myosclerosis. These issues will be described later in differential diagnosis section (Demir *et al.*, 2004; Allamand *et al.*, 2010).

1.2.1.3 *Intermediate phenotype:*

Discovery of collagen VI as disease-causing gene in both UCMD and Bethlem conditions in conjunction with improvements in diagnostic tools leads to better understanding of collagen VI-related myopathy phenotypic spectrum (Mercuri *et al.*, 2002). The term intermediate phenotype is attributed to all patients with collagen VI mutations but a transitional severity in their phenotypes. Patients in this group show a phenotype more severe than Bethlem but milder than UCMD. Intermediate phenotype patients present at birth with muscle weakness which could be severe. Also other features at birth may overlap Bethlem and UCMD. They could have severe distal hyperlaxities of UCMD at birth, whereas interphalangeal contractures of BM also exists. Muscle weakness could be stable with achieved ambulation. However, walking aids may be needed. Ambulation could be lost at later age than in typical UCMD patients or last even longer time than BM patients. Respiratory involvement is seen in intermediate phenotypes as well. Respiratory problems like other collagen VI-related patients need to be monitored (Bushby *et al.*, 2014).

1.2.2 *Prevalence of collagen VI-related myopathies:*

A prevalence of 0.13 in 100000 for UCMD and 0.77 for BM has been reported so far. With different reports of collagen VI-related myopathies from all around the world, a worldwide distribution was proposed for these disorders (Allamand *et al.*, 2010). However, new reports of collagen VI-related myopathies with different features are emerging (Clement *et*

al., 2012; Mercuri and Muntoni, 2012). An increasing number of reports of heterogeneity associated with collagen VI-related myopathy phenotypes can be found in the literature.

1.2.2.1 Prevalence of heterogenic collagen-VI related myopathies:

An emerging number of patients with collagen VI-related myopathy phenotypes have been reported that does not have any mutations in their *COL6A1-A3* genes (Bonnemann, 2011). A retrospective study of congenital muscular dystrophies' frequency in UK from 2001-2008 reported that collagen VI-related myopathy has the higher frequency (19%) then dystroglycanopathy (12%) and merosin deficient congenital muscular dystrophy (10%) (Clement *et al.*, 2012).One of the first example of collagen VI-related myopathy heterogenic patients was reported by Ishikawa. They discovered a UCMD patient with absence of collagen VI in muscle staining but no mutation was found in *COL6A1-A3* genes (Ishikawa *et al.*, 2004). In following years other reports of collagen VI-related myopathy patients with genetic heterogeneity came out (Bonnemann, 2011). However, the frequency of collagen VI-related myopathy phenotype with no mutations in collagen VI genes is not evaluated in any studies yet .In the MRC centre for neuromuscular disorders in Newcastle 50% of collagen VI-related myopathy phenotypes do not have any collagen VI mutations (Debbie Hicks, personal communication). Due to mild clinical features in some BM patients and heterogeneity in collagen VI-related myopathy which lead to undiagnosed or misdiagnosed patient, the real frequency of collagen VI-related myopathy is estimated to be higher (Clement *et al.*, 2012) .

1.2.3 *Differential diagnosis of collagen VI-related myopathies:*

As described above, collagen VI-related myopathies have a broad spectrum of clinical phenotypes. In accordance with this, the major differential diagnosis of collagen VI-related myopathies varies. Discovery of patients with Limb-girdle muscular dystrophy phenotype and myosclerosis myopathy phenotype with mutations in *COL6A1-A3* genes has led to classification of these two phenotypes as rare subtype of collagen VI-related myopathy (Jobsis *et al.*, 1996; Scacheri *et al.*, 2002; Merlini *et al.*, 2008b).

Scacheri et al (Scacheri *et al.*, 2002) in 2002 reported patients from three large families with variable limb-girdle-muscular dystrophy phenotypes. Proximal muscle weakness with onset from neonatal to adulthood was the prominent feature. Connective tissue involvement in terms of joint contractures was absent or milder than expected for collagen VI related myopathies. Patients have normal to slightly increased serum CK level with

dystrophic signs such as necrosis, fibre type variation and internal nuclei in muscle biopsies. Heterozygous missense mutations in *COL6A1* and *COL6A2* in three pedigrees were responsible for the disease in autosomal dominant mode of inheritance.

In 2008, Merlini et al (Merlini *et al.*, 2008b) reported two siblings of consanguineous parents with myosclerosis features. Progressive muscle weakness from childhood, multi-joint and Achilles tendon contractures, Slender Woody muscles –decrease in muscle bulk in the centre with long shaft muscles- with elevated CK levels were some of the clinical features in these patients. A homozygous missense mutation was identified in both siblings in the *COL6A2* gene. This mutation lead to misfolded col6a2 protein which could assemble to collagen VI monomers but the subsequent trimerization was prevented. Absence of collagen VI in immunofluorescence staining of fibroblast and muscle biopsies was seen in these patients (Merlini *et al.*, 2008b).

The above mentioned reports are two examples of collagen VI-related myopathy phenotypic variability which expand the phenotypic and differential diagnosis spectrum of these disorders. For patients with congenital onset of symptoms, other types of muscular dystrophies as well as congenital myopathies should be considered as differential diagnosis. Limb-girdle muscular dystrophy is a major differential diagnosis for the patients with adult onset symptoms and less connective tissue signs. For a classical Bethlem phenotype with joint contractures Emery-Dreifus muscular dystrophy is an important differential diagnosis (Brinas *et al.*, 2010).

Patients with proximal muscle weakness, adult-onset of the disease and mild to absent connective tissue signs could widely overlap with limb-girdle muscular dystrophy. Immunohistochemical staining, muscle biopsy which could show specific pattern of LGMD subtypes and gene screening for Limb-girdle muscular dystrophy in particular LGMD1B subtypes could help better diagnosis. One other differential diagnosis for these patients is Central core myopathy disease. This disorder is characterized by symmetrical muscle weakness, delayed motor development, congenital signs of hypotonia, joint laxity or contractures and spinal deformity. Even muscle biopsies from collagen VI-related myopathy patients could resemble central core myopathy signs with minicore-like lesions or congenital type I fibre disproportions. MRI with central core myopathy specific sign is important for right diagnosis (Bushby *et al.*, 2014).

Emery-Dreifus muscular dystrophy (EDMDs) considered to be the most important differential diagnosis for collagen VI-related myopathy disorders. This X-linked or autosomal form of neuromuscular disorder is caused by mutations in *Emerin* or *Lamin A/ C* genes. Muscle weakness and distal joint contractures are the main feature of this disorder. However, interphalangeal contractures which are found in BM myopathy are absent in EDMDs. Furthermore, cardiac involvement is very common and severe in EDMDs while it has not been reported in collagen VI-related myopathies. Patients with skin involvement are more likely to have collagen VI-related myopathies. Also, joint hyperlaxity and skin involvement are the distinct features of collagen VI-related myopathies (Bonnemann, 2011).

In infants or young children with muscle weakness and contractures, other forms of congenital muscular dystrophies which have congenital presentations should be considered. α-dystroglycanopathies, merosin-deficient muscular dystrophy or MDC1a, Fukuyama disorder or MDC1C (*FKRP* mutations), MDC1D(*LARGE* mutations),muscle-eye-brain disorders are some examples of these CMDs. Moreover, cardiac involvement, brain abnormalities revealed by MRI and highly increased CK level are some features which help to distinguish the true pathology (Bushby *et al.*, 2014).

Ehlers-Danlos syndrome for patients with joint hypermobility is also a differential diagnosis. However, skin hyperelasticity, as a sign of Ehlers- Danlos syndrome, is rarely seen in collagen VI-related myopathies. Furthermore, muscle weakness and contractures are either absent or very mild in Ehlers- Danlos syndrome compare to Bethlem myopathy (Bushby *et al.*, 2014).

As genetic and paraclinic means of diagnosis improve, the differential diagnosis for collagen VI-related myopathies will be broader. In 2013, our team together with Cossins et al (Cossins *et al.*, 2013) reported novel genes*ALG2* (Asparagine-linked glycosylation 2) and *ALG14* (Asparagine-linked glycosylation 14) in three families with large pedigrees. Two of these families with three affected individuals had been treated for congenital myasthenic syndrome in Oxford. They show an autosomal dominant pattern of inheritance for two mutations in *ALG2* and *ALG14* gene, respectively*. ALG2* is a member of post-translational glycosylation family. Other members of this family such as*DPAGT1* have been identified in myasthenic patients as disease-causing genes (Belaya *et al.*, 2012). The later family was a large family with four affected siblings and unaffected parents. This

Saudi Arabian family shows an autosomal recessive pattern of inheritance and had been referred as Ullrich congenital muscular dystrophy phenotype with no mutations in *COL6A* genes in the Centre for Neuromuscular Disorders in Newcastle. All four affected individuals had a history of hypotonia, muscle contractures and delayed motor milestones during infancy or the first year of life. Ambulation was lost in four affected siblings. Respiratory insufficiency was seen with variable severity in all four affected individuals. No cardiac or skin involvements was seen. Muscle weakness had a progressive pattern in proximal muscle without fluctuation. No history of ptosis was recorded. Previous evaluation did not reveal any mutations in *COL6A* genes and immunofluorescence staining for collagen VI in fibroblast of patients was normal. Autozygosity analysis identified a region of 27 MB in chromosome 9 (9q31.1) as the homozygous candidate interval. Whole-exome sequencing was used to identify mutations in this candidate interval. With filtering algorithm less likely pathogenic variants based on their frequency (>1%) and prediction to be non-pathologic were excluded. The resulting 48 candidate variants were investigated through Sanger sequencing. One variant c.214-226delGGGGACTGGCTGCinsAGTCCCCCGGC p.72-75delGDWLinsSPR was identified in *ALG2.* Figure 1.4 shows this variant in the family.

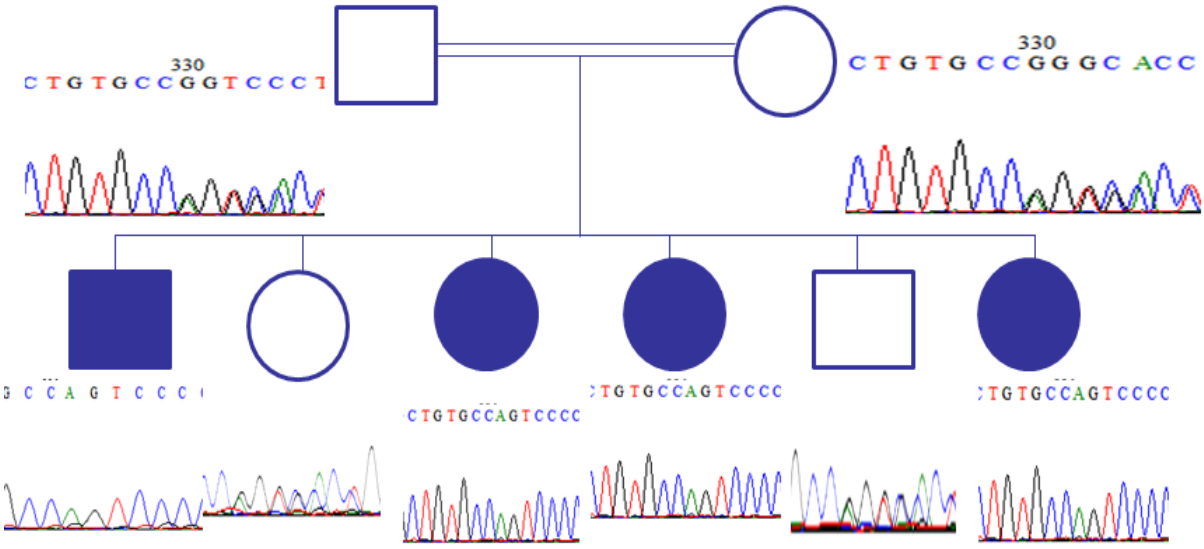


Figure 1.4. Asparagine-link-glycosylation2 (*ALG2*) segregation in a large family of Ullrich like phenotype. An indel in the gene Asparagine Link Glycosylation-2 **(*ALG2* exon1 NM.033087.3:c.283-296delGGGGACTGGCTGCc.283-293insAGTCCCCGGC p.73-76delGDWLinsSPR)** was found which completely segregated with UCMD in corresponding pedigree. Affected individuals are homozygote for this variant while unaffected individuals are heterozygote for the variant. The variant acting as recessive.

Retrospective clinical evaluation which revealed signs of myasthenic syndrome as facial weakness and repetitive nerve stimulation (RNS) which showed an increase in two examined patients led to a diagnosis of congenital myasthenic syndrome in these patients (Cossins *et al.*, 2013).

The above mentioned case, as an incidental finding during our research for novel genes in collagen VI-related myopathy phenotypes has broadened the spectrum of these disorders differential diagnosis.

1.2.4 *Pathogenesis of collagen VI-related myopathies:*

Collagen VI is known to be responsible for around 50% of collagen VI-related myopathies (Debbie Hicks, personal communication). Collagen VI is a heterotrimeric monomer mainly consisting of three α-chains: α1, α2 and α3. These chains are encoded by *COL6A1*, *COL6A2* and *COL6A3* genes.The first two genes are on chromosome 21q22 and *COL6A3* is on 2q37. A short chain of 331-336 amino acid is the backbone of each of the collagen VI α-chains. Figure 1.5 shows collagen VI chains and their assembly (Bushby *et al.*, 2014).

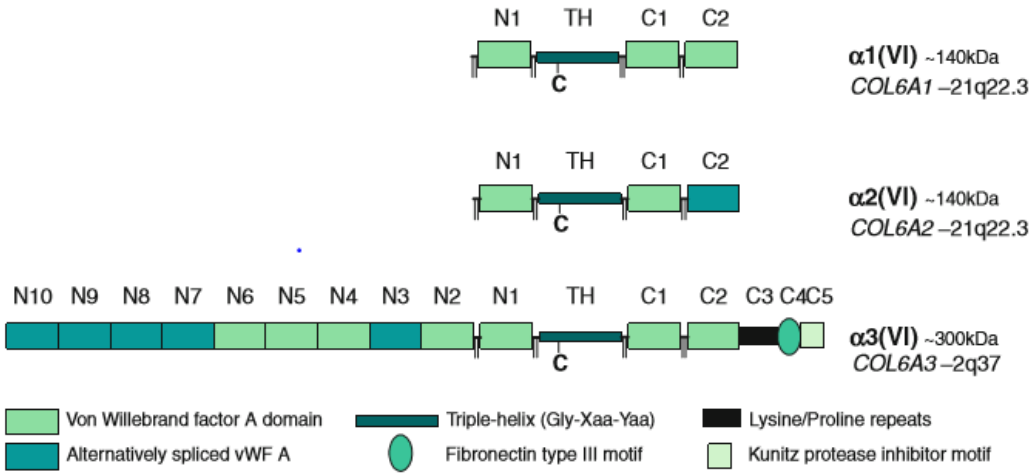


Figure 1.5.Collagen α1 (VI), α2 (VI), α3 (VI) polypeptide chain structure. Three α chains encoded by *COL6A1, COL6A2* and *COL6A3* genes. Each chain comprises of von wilebrand factor A domains and fibronectin type III domains. Cysteine residue which is crucial for collagen VI dimerization and tetramerization is demonstrated in each chain From (Lampe and Bushby, 2005)

Collagen molecules undergo different steps of assembly. A cysteine residue is always placed in the N-terminal part of each collagen chain. This residue is necessary for

intracellular assembly and subsequent dimerization and tetramerization of collagen VI. Collagen (α1) VI and collagen (α2) VI have similar structures with two possible C-terminal and one N-terminal von wileberand globular A domain. C-terminal undergoes post-translational assembly and splicing. For example *COL6A2* has two spliced C-terminals. The C1is enough for monomerization but C2 is important in post-translational assembly Collagen (α1) VI and collagen (α2) VI chains are likely to derive from duplication of a common ancestral gene. Collagen (α3) VI chain has one large and spliced N-terminal domain with one C-terminal domain. The distinct role of these splice variants is unknown but the truncated splice variants (with any affected domains) seem to have lesser effects than non-spliced ones (Demir *et al.*, 2004).

Three additional *COL6A* genes, *COL6A4*, *COL6A5* and *COL6A6* have been identified in mouse. Two of these genes, *COL6A5* and *COL6A6* are also present in humans, while *COL6A4* is interrupted by and is not functional. *COL6A5* and *COL6A6* are able to substitute for *COL6A3* during collagen VI trimerization. However, no patients with mutations in these genes have been reported so far (Fitzgerald *et al.*, 2008; Bushby *et al.*, 2014).

COL6A1, COL6A2 and mainly *COL6A3* combine to make a collagen VI monomer. This trimeric then undergoes a complicated assembly process which starts from C-terminals. Gly-X-Y motifs in the C-terminus play important role to establish the monomerization. In this motif, One glycine is repeated in every three amino acids. X and Y represent two other amino acids. N-terminal cysteine in α1 and α2 chain should interact with C-terminus globular end cysteine of another α chains in order for two collagen VI monomer twitch to each other in an antiparallel arrangement. Two collagen VI dimers are associated to each other in parallel direction to form a tetramer. Thus, dimers of collagen VI as well as tetramers are stabilized with disulfide bonds. Collagen VI is secreted to the extracellular matrix as tetramers. These tetramers then align end to end to form microfibrils. These microfibrils of collagen VI in extracellular matrix have the periodicity of 100-105nm and 4.5 nm diameter (Bonnemann, 2011). Figure 1.6 shows collagen VI molecules assembly.

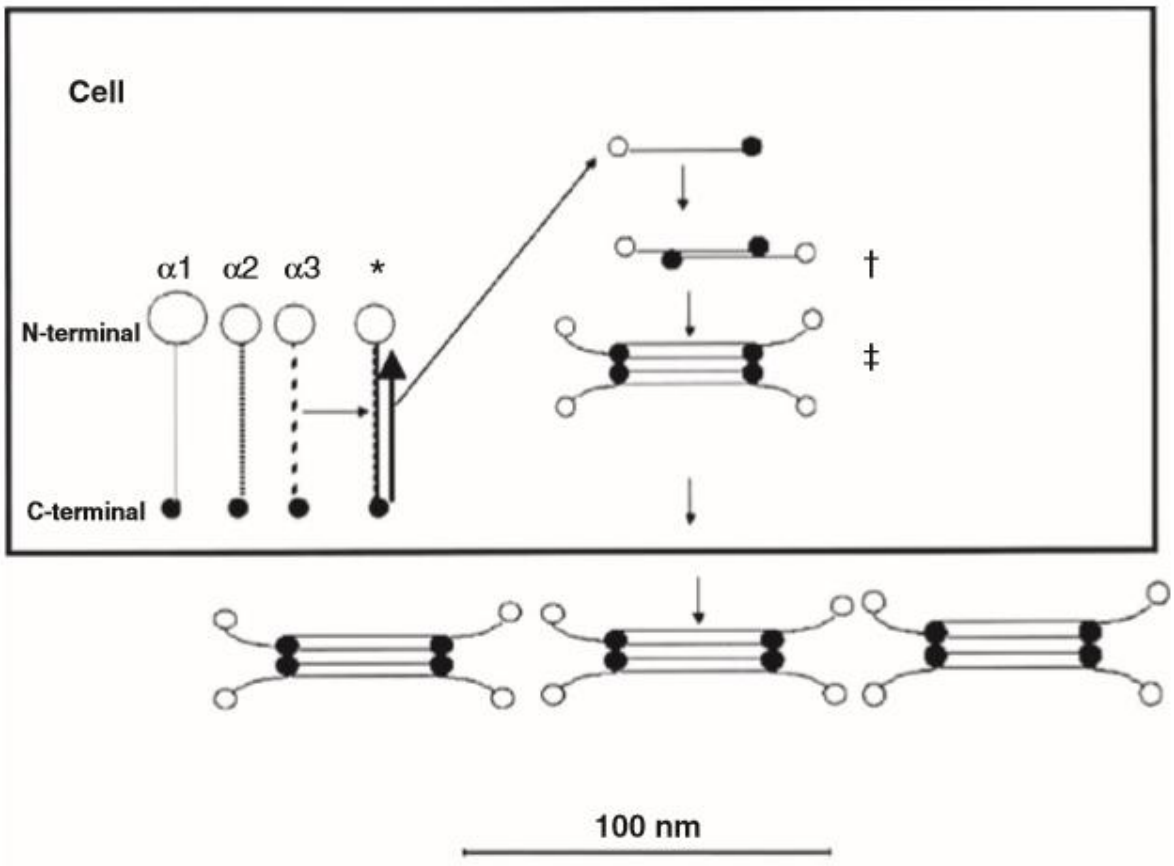


Figure 1.6.Schematic picture of collagen VI molecule assembly. Upper figure shows intracellular assembly from the heterotrimeric monomer composed of all three a chains (*), via antiparallel dimer (†) to tetramer (‡) formation. The lower picture shows the formation of the beaded collagen microfilaments with 100-nm periodicity of tetramers, These microfilaments formed by close interaction of the C- and N-terminal globular domains (Bonnemann, 2011).

Collagen VI is found in many tissues and organs such as muscle, tendon and endothelial part of vessels, cornea, eye, skin and cartilage. In these tissues, collagen VI is localized in the extracellular matrix material called basement membrane (Bonnemann *et al.*, 2011). The basement membrane in muscle tissue is neighbouring muscle fibres. It was believed that the basement membrane had only anatomical role in muscle structure. However, recently it has been found that the basement membrane is an active structure with signalling, synaptogenesis and myogenesis roles (Bonnemann, 2011). Therefore, mutations in basement membrane proteins such as collagen VI are responsible for some of the muscular dystrophies. Other type of neuromuscular disorders are caused by mutations in the dystrophin-glycoprotein complex (DGC) which comprises crucial proteins for muscle function and integrity. DGC is a complex of glycoproteins and oligomeric proteins which localizes to the sarcolemmal membrane and mediates linkage of actin

filament to extracellular matrix and maintains muscle membrane stability (Bushby *et al.*, 2014). Figure 1.7shows a schematic picture of DGC complex.

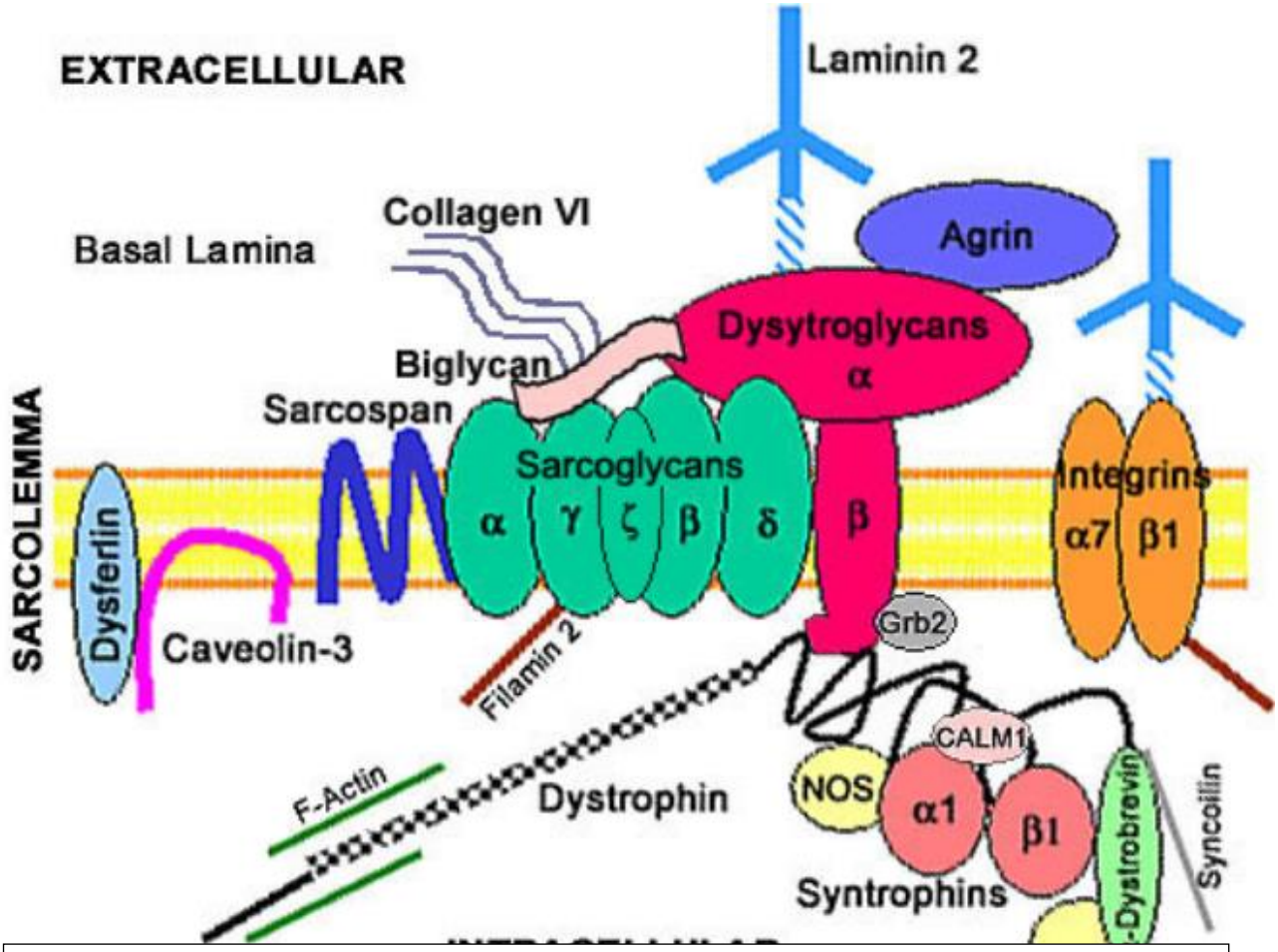


Figure 1.7. Schematic picture of DGC complex. Different molecules in DGC complex and their interaction was depicted in schematic view. Collagen VI is an extracellular molecule in this complex (https://neuromuscular.wustl.edu/musdist/dag2.htm).

In addition, collagen VI is located around the pericellular space of tendons and nerves. Immunofluorescence staining shows the affinity of collagen VI for other basement membrane molecules such as collagen type IV, perlecan and laminiɣ1. Collagen VI in the extracellular matrix mostly interacts with collagen II and collagen IV fibrils and possibly collagen I (as the most abundant type of fibrils), fibronectin, fibulin-2, perlecan, decorin, byglycan, heparin and hyaluran. Collagen VI also binds to cell surface of CD44, NG2 and integrin. The specific receptor for collagen VI in muscle cell is unknown (Bonnemann, 2011).

The exact pathomechanism for collagen VI-related myopathy is unknown. It has been suggested that collagen VI could play roles in proliferation, adhesion and DNA synthesis for mesenchymal cells and neural crest. Research reveals an absence of collagen VI from basal lamina in UCMD patients. In these patients basal lamina has also lost attachments to extracellular matrix. These findings suggest a role for collagen VI in basement membrane attachments (Ishikawa *et al.*, 2002). Furthermore, collagen VI plays a role in fibronectin organization in fibroblasts cells. Without collagen VI anchoring fibronectin in ECM, the extracellular matrix might be disorganized (Bushby *et al.*, 2014). However, the role of collagen VI in myogenic tissue is unknown. It might be that collagen VI affects myogenic cells through an indirect pathway, because collagen VI is secreted from interstitial fibroblasts in muscle tissue (Zou *et al.*, 2008). It is suggested that collagen VI induces myogenic cell DNA synthesis and proliferation. Furthermore, there is a possibility of direct link between collagen VI and muscle matrix via NG2 (Petrini *et al.*, 2005). Several studies suggest that collagen VI could influence apoptosis in cells through relatively unknown pathway (Irwin *et al.*, 2003; Petrini *et al.*, 2005)

Our understanding of collagen VI-related myopathies has developed via animal modelling. Homologous inactivation of *COL6A1* locus helped to generate a mouse model of collagen VI deficiency. This mouse shows very mild neuromuscular phenotype, in contrast to patients with complete collagen VI α1 chain deficiency who show severe UCMD signs. However, myopathic signs such as necrosis, phagocytosis and variation in fibre type diameter were reported from collagen VI deficient mouse. Furthermore, contractile strength is reduced in this mouse which could be associated with ultrastructural disorganization of sarcoplasmic reticulum or mitochondria (Bonaldo *et al.*, 1998). Mitochondrial permeability transition pore (PTP) in *COL6A1-/-* null mouse had dysfunction which led to early breakdown of mitochondrial potential in mitochondria pre-challenged with oligomycin (Irwin *et al.*, 2003). This PTP dysfunction is under influence of cyclophilin D and with adding wild type collagen VI or cyclophilin D inhibitor cyclosporine A could be corrected (Palma *et al.*, 2009). Collagen VI-cyclophilin D double-knockout mouse has prevented PTP dysfunction and decreased muscle apoptosis which confirms cyclophilin D role in PTP dysfunction (Palma *et al.*, 2009). Increased apoptosis was reported also in fibroblasts cell cultures from collagen VI-related myopathy patients and muscle biopsies of patients (Merlini *et al.*, 2008a). In addition, electron microscopy of *COL6A2* affected individuals' skin biopsy reveals abnormality in mitochondria (Zamurs *et al.*, 2015).

However, PTP dysfunction is not specific of *COL6A1* -/- mouse and was also seen in merosin-deficient myopathy (Millay *et al.*, 2008; Hicks *et al.*, 2009a). Thus, more studies in this field is needed to reveal potential relation between apoptosis pathway and collagen VI deficiency (Bushby *et al.*, 2014). In *COL6A1* null mouse mitochondria and sarcoplasmic reticulum have altered morphology as well (Bonaldo *et al.*, 1998). Another observation about *COL6A1* deficient mouse is its impaired autophagic function. This suggests a relation between mitochondrial dysfunction and muscle degeneration. Inducing autophagy in collagen VIα1 deficient mouse could ameliorate its symptoms which opens a way for therapeutic method in collagen VI-related myopathy patients (Bushby *et al.*, 2014).

A zebrafish model of collagen VI-related myopathy which used morfolino approach to knock down *COL6A1* and *COL6A3* genes, has shown PTP dysregulation and increase apoptosis rate as well as impaired muscle development (Telfer *et al.*, 2010).

1.2.5 *Phenotype/ Genotype correlations:*

So far, a large number of mutations in *COL6A1*, *COL6A2* and *COL6A3* have been reported in collagen VI-related myopathies. However, this should bear in mind that lack of mutations in *COL6A1*, *COL6A2* and *COL6A3*do not exclude the possibility of Bethlem or Ullrich congenital muscular dystrophies (Bushby *et al.*, 2014).

In 2010, Allamand et al (Allamand *et al.*, 2010)reported more than 35 mutations inherited as autosomal recessive in 30 families and 38 mutations acting as dominantly negative in 58 autosomal dominant patients. In Bethlem myopathy, 45 dominant negative mutations has been identified in 65 Bethlem families (Allamand *et al.*, 2010).As the number of reported mutations in collagen VI-related disorders increase, genotype-phenotype correlation studies begin to emerge. We describe common genetic mutations and mode of inheritance for Bethlem and Ullrich congenital muscular dystrophys below.

1.2.5.1 *Bethlem myopathy:*

BM is mostly reported as an autosomal dominant disorder, with mutations acting as dominantly negative in one allele inherited from a parent or emerged as *de novo*. Mutations in Bethlem myopathy could be classified in three types. The most common form is mutations in Gly-X-Y motif in N-terminal end of collagen microfibrils (Brinas *et al.*, 2010). Lamande et al (Lamande *et al.*, 2002) reported that this type of mutations may cause a kink in the collagen VI tetramer which is responsible for the dominant negative effect of the

mutations (Lamande *et al.*, 2002). However, these mutations have different severity in clinical phenotypes based on the effect of mutation in protein assembly. The more severe ones fall into UCMD category (Bonnemann, 2011).

The second most common mutation in Bethlem myopathy is in frame deletion of exon 14 in collagen α1 (VI) chain. This mutation results in a lack of one cysteine which is important for the dimer assembly. Because the covalent attachments which stabilizeing dimers form between parallel cysteins. The more severe forms of these mutations which could inhibit monomerization, could also cause UCMD (Pan *et al.*, 2003). The third most common mutations in BM patients are those which do not affect triple helical domain of protein. These types of mutations are mostly seen in patients with the Limb-girdle muscular dystrophy pattern (Scacheri *et al.*, 2002). In 2009, Foley et al (Foley *et al.*, 2009) reported a case of autosomal recessive Bethlem myopathy. The two affected individuals were compound heterozygous for a single nucleotide deletion (exon 23; c.1770delG), which caused in-frame deletion of exon 23in one allele (maternal) and a missense mutation in the other allele (paternal) of *COL6A2*. While Homozygosity for the null allele inhibited collagen tetramerization, the affected mother do not show any symptoms. It seems that the missense mutation from paternal allele could increase pathogenicity, and the affected heterozygous siblings represent Bethlem phenotype (Foley *et al.*, 2009). The similar mechanisms of compound heterozygosity was described by Gualandi et al with one truncated mutation and one missense mutation in *COL6A2 (Gualandi et al., 2009).*

1.2.5.2 *Ullrich congenital muscular dystrophy:*

The first UCMD mutation described were null mutations in *COL6A2* inherited in an autosomal recessive pattern and that to absence of collagen α2 (VI) in patients' muscle biopsy (Camacho Vanegas *et al.*, 2001). Subsequently, a large number of mutations which led to premature stop codon in collagen VI α1, α2 and α3 have been reported. All these mutations are inherited in an autosomal recessive pattern and cause an absence or significant decrease in the amount of protein in immunohistochemical evaluation. The ones that located in alternatively spliced regions always show milder symptoms(Lampe and Bushby, 2005). Furthermore, haploinsufficieny in one of collagen VI chains does not affect the protein structure and many of heterozygote individuals with these mutations are healthy carriers. Splice-site mutations which lead to out-of-frame exon skipping or in frame

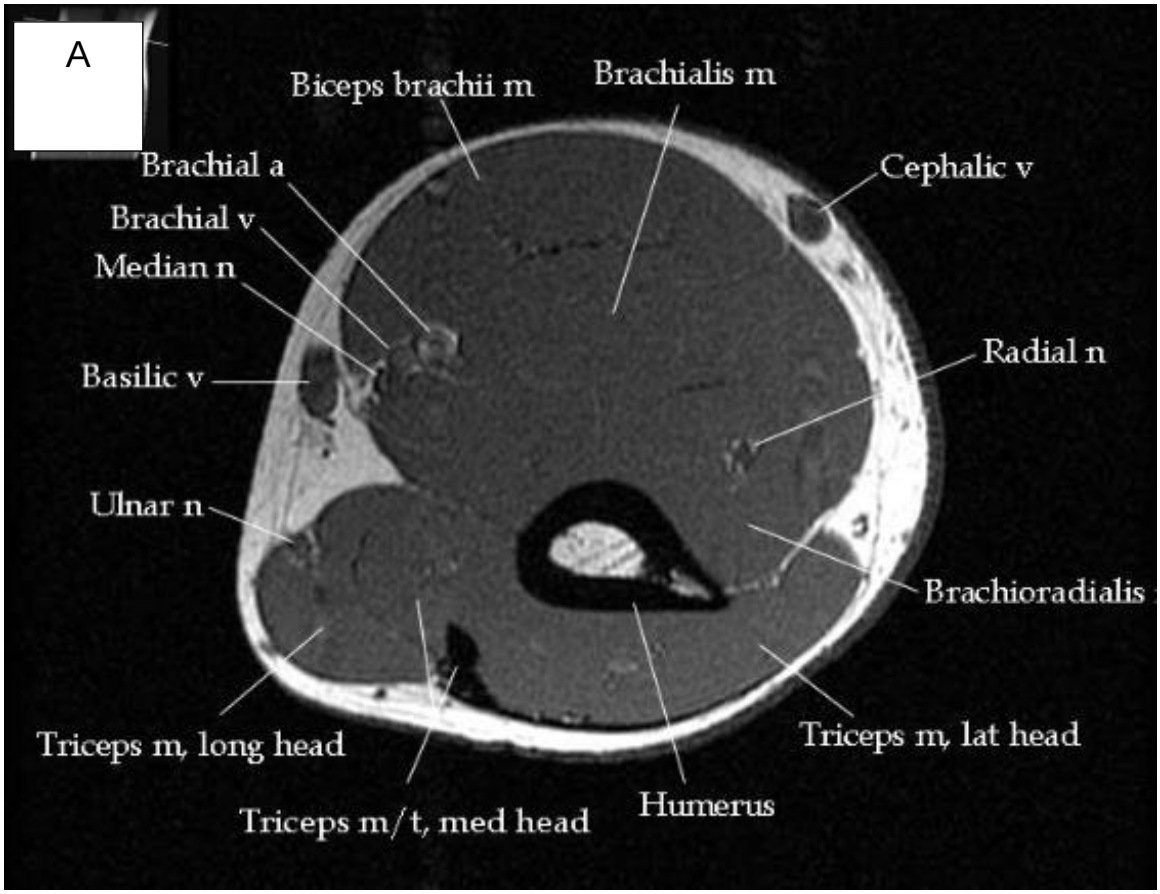
exon skipping in C-terminal of the protein are another example of UCMD recessive inheritance (Bonnemann, 2011).

As mentioned above the number of mutations acting as dominant negative in UCMD is almost equal to recessive mutations. *De novo* mutations which cause in-frame exon deletion and often acting as dominant negative are responsible for high proportion of dominant UCMDs (Allamand *et al.*, 2010). These mutations always cause exon deletion in N-terminal part of the collagen chain. Thus, collagen chain could assemble to dimers and tetramers but spare cysteins in these parts affect higher-order assembly in mutant chains. Generally, UCMD phenotypes associated with dominantly acting mutations are more likely to present milder symptoms and achieved ambulation during infancy. However, the course of the disease is still progressive (Foley *et al.*, 2011). While heterozygous dominant negatively-acting mutations and recessively-acting loss-of-function mutations are the two common way of inheritance in UCMDs, in 2011, Foley et al (Foley *et al.*, 2011) described large genomic deletions on chromosome 21q22.3 which is inherited as autosomal recessive in two families. Clinically unaffected parents had large genomic deletions of *COL6A1* and *COL6A2* which could obscure the true genetic causation of the disease in non-deleted allele when it inherited hemizygously. These unaffected individuals provide evidence that haploinsufficiency for *COL6A1* and *COL6A2* is not a disease mechanism for Bethlem myopathy. Single nucleotide polymorphisms (SNP) array was used to detect the deletion that included *COL6A1* and *COL6A2* with adjacent genes (Foley *et al.*, 2011).In 2010, bovolenta et al (Bovolenta *et al.*, 2010) represented a GCH system to detect copy number variants in coding and non-coding regions of *COL6A1-A3* genes and other related genes. They investigate a cohort of 12 BM patients with this system and find a deep intronic mutation in *COL6A2* gene in BM patient. This mutation from a non-affected parent when accompanied with polymorphism from another unaffected individual led to BM symptoms (Bovolenta *et al.*, 2010). It is important to remember single nucleotide sequencing could not detect such mutations in patients and more complicated modes of mutation segregation in collagen VI-related myopathy remain to be elucidated.

1.2.6 *Collagen VI-related myopathy diagnosis strategy:*

After considering the clinical presentation, collagen VI-related myopathy may include in differential diagnosis table for a neuromuscular patient. There are laboratory, imaging and genetics steps which help to confirm Collagen VI-related myopathy diagnosis.

Serum creatine kinase level as one cheap and available laboratory test is measured in neuromuscular disorders. CK in collagen VI-related myopathy can be normal, slightly elevated or highly elevated (up to five times). CK could go back to normal levels during course of the disease (Bushby *et al.*, 2014). Magnetic resonance imaging (MRI) of limb muscles is another diagnostic method in BM and UCMD patients. Imaging in these patients reveals a picture of connective tissue and fat replacement in muscle which is focused around the fascias of muscle. This pattern is mostly seen in tight muscle such as rectus femoris and vastus lateralis (Mercuri *et al.*, 2002). The periphery of the involved muscle has markedly increased signals with central sparing. Central shadow in these patients describes as increased signal in the anterior part of rectus femoris is mostly focused around central facsia. MRI of calf muscles represents diffuse and increased signals of fatty infiltration in soleus, gastrocnemius, tibialis anterior and popliteous muscle (Mercuri *et al.*, 2003). Figure 1.8 represents muscle MRI in patients.



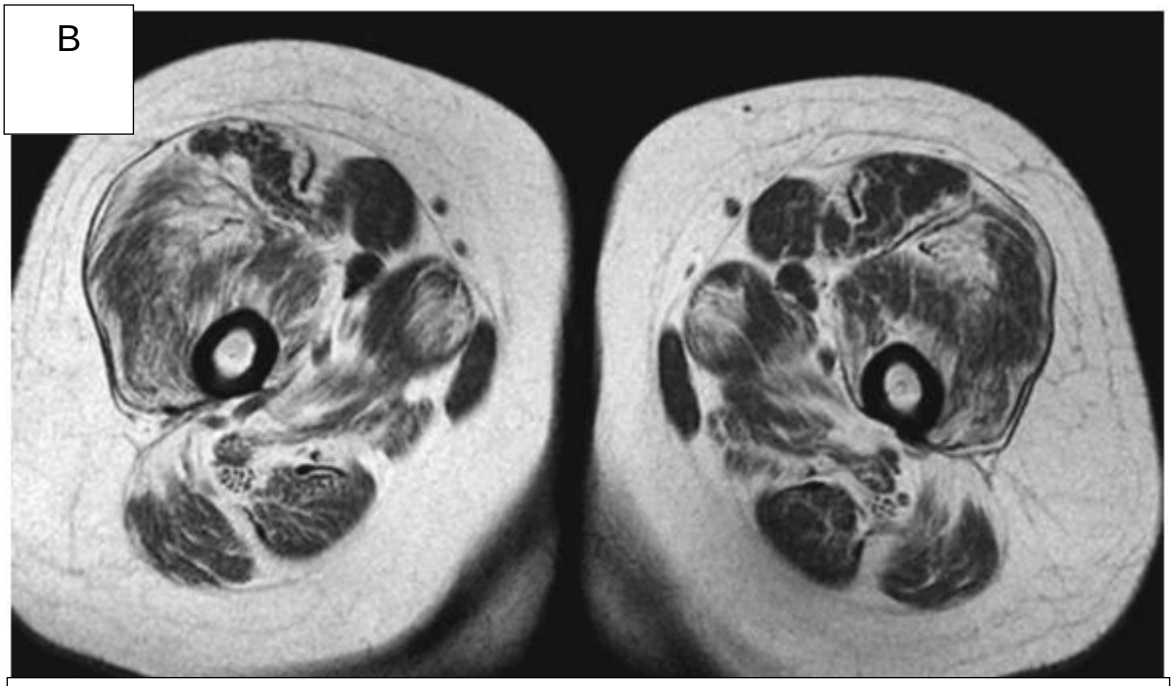


Figure 1.8. MRI features in collagen VI related myopathy. A) MRI of normal tight muscle. B) Diffuse pattern of involvement with relative sparing of the Gracilis and Sartorius muscle. A rim of abnormal signals is visible around muscle periphery with relative central sparing. Signal intensity is in the central part of muscle markedly increased (Bushby *et al.*, 2014).

Electromyography (EMG) is another method specified for neuromuscular disorders. Pathologic changes of muscle demonstrate in EMG as reduced number of high-amplitude polyphasic motor unit potentials and prolonged insertional activity (Allamand *et al.*, 2010).

Muscle biopsy is another method for differentiating different types of muscle disorders. Muscle biopsy in UCMD demonstrates common dystrophic features of replacement of muscle fibers with fibrous connective tissue, fatty infiltration, fibre diameter variation and muscle degeneration signs. In Bethlem myopathies muscle biopsy could be variable and shows normal to mild myopathic features. These features include muscle fibre disproportion and fibre atrophy. These features are ameliorating with age. Muscle biopsy sometimes mimics core-muscle myopathy signs which could cause false diagnosis (Bonnemann *et al.*, 2011). Immunohistochemical studies in muscle for collagen VI show a spectrum of absent to moderately or markedly reduced signals around basal lamina that could be normal for endomysium. In severe cases with normal immunofluorescence staining of collagen VI in fibroblast, a dual- labelling of collagen VI with a basement membrane marker as perlecan or collagen IV is helpful. In these cases proper localization of collagen VI was lost because the secreted protein could not assemble to extracellular matrix. In milder cases this detachment from basal lamina could be partial or muscle immunolabeling present as normal. Figure 1.9 and 1.10 demonstrates immunohistochemical staining of muscle in patients (Bonnemann *et al.*, 2011; Bushby *et al.*, 2014).

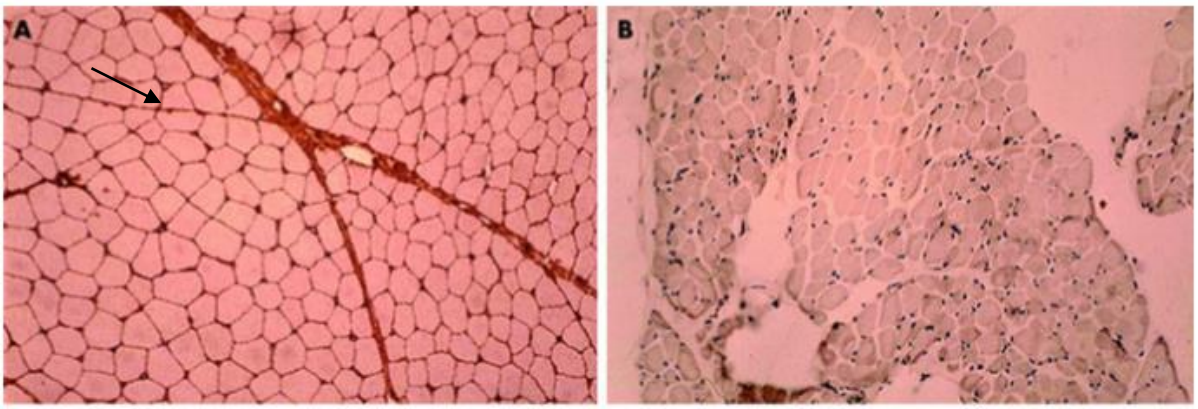


Figure 1.9.Immunohistochemical staining of collagen VI in muscle biopsies of normal and mutant individuals. Collagen VI is absent from basement membrane of muscle biopsy of an individual with collagen VI related myopathy (B) compare to control (A). Collagen Vi signals depicted in red and demonstrated with black arrow pointed basement membrane in control panel (Bushby *et al.*, 2014).

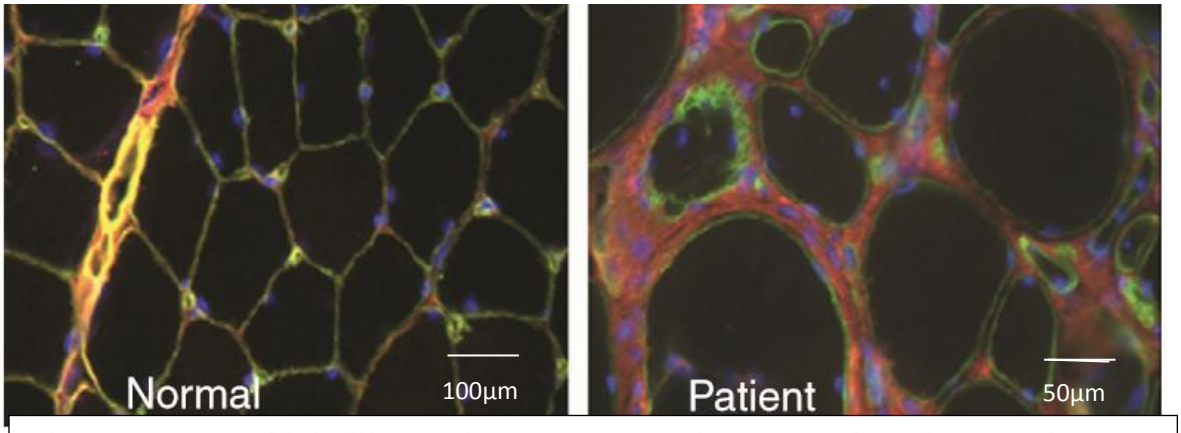


Figure 1.10.Dual labelling of basement membrane markers in muscle biopsies of normal and mutant patients. Detachment of collagen VI (red) from basement membrane marker (green) is demonstrated in patients compare to normal biopsy. In normal biopsy attachment lines (yellow) are demonstrated as combination of collagen VI(red) and basement membrane marker(green) (Bonnemann, 2011).

Because of limitations for accessing muscle biopsies, immunohistochemical staining in dermal fibroblast from collagen VI patients has been considered in several studies (Jimenez-Mallebrera *et al.*, 2006; Hicks *et al.*, 2008). Hicks et al (Hicks *et al.*, 2008) reported that immunofluorescence staining for collagen VI in dermal fibroblast from patients is a sensitive and specific method to diagnose collagen VI subtle abnormalities. 78% of genetically confirmed Bethlem patients reported to demonstrate abnormalities in immunofluorescence staining. They suggested to use immunofluorescence staining as predictive tool to guide genetic test for collagen VI-related myopathy phenotype (Hicks *et al.*, 2008). Figure 1.11 shows possible changes in immunofluorescence staining of collagen VI in Bethlem patient fibroblasts.

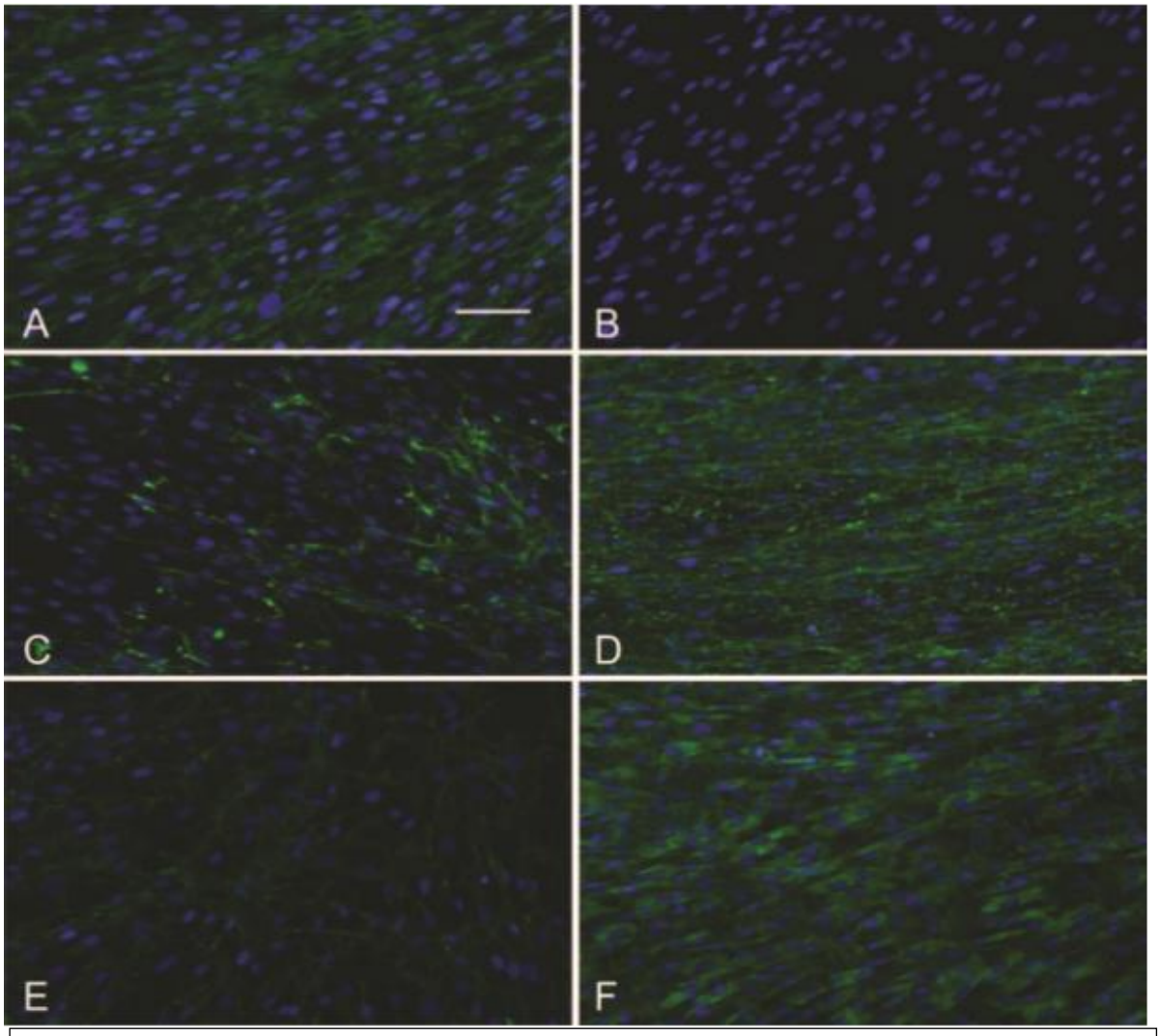


Figure 1.11.Immunofluorescence staining of fibroblasts for collagen VI in collagen VI-related myopathy. Immunofluorescence staining of collagen VI (green) in fibroblasts of normal individuals which presents well0organized, linear and unidirectional pattern in normal individual (A). Negative control with cell nuclei presents in blue (B). Immunofluoresence staining of BM patient fibroblasts which present disruption in unidirectional collagen VI microfibrils (C). Stippling pattern in BM patient (D), rearification pattern (less collagen VI is observed) in BM patient (E), Intracellular retention pattern which collagen VI can only observed when cell membrane permeabilized with TritonX100 in BM patient (F) Scale bar=100um (Hicks et al., 2008).

Genetic tests are widely believed to be the gold-standard for Bethlem myopathy diagnosis. Genetic analysis sequences all exons in three *COL6A (A1, A2, A3)* genes (Lampe et al., 2005). However, in order to confirm pathogenicity of a genetic variation, inheritance pattern of that variation in the corresponding pedigree should be investigated (Bonnemann, 2011). Analysing collagen VI production in dermal fibroblasts with immunofluorescence staining that could show signal reduction due to less protein

production, to identify splice-site variation in patients with no mutations in collagen VI is a helpful method (Lampe *et al.*, 2008). Exon sequencing is not suitable method for large exonic deletions. For example, large exonic deletions of *COL6A1* and *COL6A2* have been reported to be missed during exome sequencing (Foley *et al.*, 2011). In order to detect such variation, the increased use of chromosomal micro-array platforms including those based on single nucleotide polymorphism (SNP) is seen in novel reports. These methods also leads to an increasing catalogue of genomic deletion and duplication variants of unknown significance (Bonnemann, 2011; Foley *et al.*, 2011).

1.2.7 *Available treatment and management of collagen VI-related myopathy:*

Currently available therapeutic strategies for collagen VI-related myopathy mainly focus on managing various clinical presentations of these disorders as well as improving quality of life. During neonatal period, for severe cases, therapeutic interventions should provide non-invasive treatments for respiratory problems due to chest muscle weakness or feeding reflux. Contractures are primarily addressed by stretching program in conjunction with dynamic splint. In severe cases, surgery to release contractures for example in Achilles tendon should be considered. However, there is a possibility of contractures recurrence after surgery as well as limited effect on flexor contractures especially in fingers. Brace as the first step of scoliosis management is implemented. New scoliosis surgery techniques also may apply in special conditions. For adult respiratory problems, upright positioning and bilevel positive airway pressure are beneficial. Furthermore, regular follow-ups for adult with respiratory involvement are necessary to prevent high speed progression of the disease and update clinicians about respiratory status. Ventilation support could stabilize patient's condition for long time. Calcium and vitamin D are two supplementary drugs which prescribed to maintain bone marrow density in collagen VI-related myopathies. Feeding problems due to muscle weakness is another problem. Appropriate diet for Bethlem patients as well as supportive device and surgery ,like percutaneous gastrostomy tube, is helpful (Bonnemann, 2011).

To date, several pharmacological and molecular approaches have been investigated in collagen VI- related myopathies. As fiber apoptosis has previously identified in collagen VI-related patients, antiapoptotic drugs as possible therapeutic strategies are implemented in animal model and clinical trials. Cyclosporin A in one clinical trial with five patients was reported to be beneficial for reducing apoptosis and improving mitochondrial permeability

transition pore(PTP) but had no effect on muscle strength (Merlini *et al.*, 2008a; Merlini and Bernardi, 2008)

Apart from a pharmaceutical approach, several genetic approaches were evaluated in collagen VI-related myopathies. Various modes of inheritance and different pathogenic mechanism for BM and UCMD are two important challenges for developing a genetic approach for collagen VI-related myopathies. Using siRNA to silencing mRNA mediated decay pathway in one UCMD patient with *COL6A2* null mutation was reported to increase amount of collagen α2 (VI) chain and even reduce the symptoms (Usuki *et al.*, 2006). In addition, siRNA allele specific silencing to knockdown genes with dominant negative mutations has been tested in *COL6A3*alleles. It has been shown that this approach could increase collagen VI deposition in fibroblasts and improve its pattern in a mouse model(Bolduc *et al.*, 2014).

1.3 Extracellular matrix structure and composition:

Extracellular matrix (ECM) is part of connective tissue, which is found in tendon, ligaments, bone and different organs. The extracellular matrix is composed of three main elements: proteoglycans, various glycoproteins and collagens (Halper, 2014b). Collagens, as the most abundant proteins of human body, will be described in detail later. Proteoglycans which are divided into two subtypes, large and small molecules, are an important component for assembly and regulation of extracellular matrix. These proteins play a role in cell proliferation through their interactions with different growth factors. Small proteoglycans or leucine-rich proteoglycans (SLRPs) are sub-classified into class I-V. Various members of class I and II such as decorin, biglycan, fibromodulin and lumican bind to collagens and share attachment sites. They could also substitute for each other in animal models (Iozzo, 1998).

Aggrecan and Versican are two examples of large proteoglycans. The two molecules mainly localize in the cartilage, where they act as resilience provider. They also play a role in tendon strength. Mutations in large proteoglycans could lead to heart deformity and dysfunction (Iozzo, 1998).

Glycoproteins are another type of structural and signalling proteins in the extracellular matrix. Several glycoproteins have been identified acting as structural or mediating signals during developmental phase of extracellular matrix. Other roles of glycoproteins include

their roles in normal tissue physiology, tissue homeostasis, responding and adapting to environmental changes such as mechanical loading/unloading and tissue damage or regeneration. In addition, during cancer, tissue fibrosis and anomalies, glycoproteins are involved in tissue response. Glycoproteins expression could be modulated by mechanical stress and mechanical loading on specific tissue. Collagens and glycoproteins also have similar expression patterns (Halper and Kjaer, 2014). The most important glycoproteins in extracellular matrix structure will be described below.

1.3.1 *Collagens:*

Collagens are the most common proteins in connective tissue and the extracellular matrix. All 28 collagen molecules are trimers which are composed of at least one collagenous domain (C) as well as several non-collagenous domains (NC).The collagens are classified according to their domain structure and supramolecular organization. Each collagen is given a roman number (I to XXVIII) in chronological order of discovery. Forty-five different genes encode these 28 collagens. α chains are structural unit of collagens. Each α chain is different in its structure and is encoded by a different gene. Moreover, each α chain has distinct primary structure which is unique in amino acid sequence and domains. α chains are forming trimers which are an assembled form of collagen primary structure secreted to ECM. Collagen trimers are either homotrimer which means they are composed of three identical α chains or heterotrimer which means they are composed of three different α chains encoded by three different genes. Collagen I is a homotrimer while collagen VI is a heterotrimer (α1, α2 and α3). Some heterotrimeric collagens, such as collagen V have more than one chain composition. Collagens could be grouped according to their suprastructural organizations(Halper, 2014a). Table 1.1 shows the collagen classification.

Classification	Collagen type	Supramolecular organization
Fibril-forming collagen	I,II,III	Striated fibrils, Striated fibrils retained N-terminal domains
	V, IX	
	XXIV, XXVII	
FACIT collagens	IX, XII, XIV	Associated with fibrils, other interactions
FACIT-like collagens	XVI, XIX, XXI, XXII	Interfacial regions, Basement membrane zones
Basement membrane	IV	Chicken wire network with lateral association
Transmembrane collagens	XIII, XVII, XXIII, XXV	Transmembrane and shed soluble ectodomains
	Gliomedins, ectodysplasin	
Beaded-filaments forming	VI	Beaded filaments, networks
Anchoring fibrils	VII	Laterally associated anti-parallele diameter
Hexagonal networks	VIII, X	Hexagonal lattices
Multiplexin collagens (Endostatin–XV and XVIII)	XV, XVIII	Basement membranes, cleaved C-terminal domains influence angiogenesis
Other molecules with collagenous domains	XXVI, XXVIII	Collagenous domains in primarily non-collagenous molecules
	Acetylcholinesterase, adiponectin, C1q, collectins, surfactant protein	

Table 1.1. General Classification of different collagen types. Modified from (Halper and Kjaer, 2014).

Collagen VI which has been described before is classified as beaded fillament-forming collagen. Collagen VI suprastructure can have different forms included beaded fibrils, hexagonal structure or broad banded structure (Wenstrup *et al.*, 2011). Figure 1.5 shows different forms of collagen VI previously.

Collagen VI interacts with many molecules including collagen I, IV and XIV, decorin, biglycan, heparin, fibril associate glycoproteins (MAGP1). Through its wide distribution and several binding partners, collagen VI is proven to have different roles. Like other collagens, collagen VI interactions` with various molecular complexes could affect its suprastructure. For example, biglycan interactions with collagen VI tetramers could induce hexagonal lattice but decorin is less likely to induce hexagonal form. It should be remembered that collagen VI different functions are applied through its various forms. These different forms could simply add or remove small non-collagenous molecules in extracellular matrix. Collagen VI is essential in muscular extracellular matrix as its lack leads to collagen VI-related myopathy disorder. Furthermore, it is important in tendons and ligaments (Izu *et al.*, 2012).

In the *COL6A1-/-* null mouse model, tenocyte expression is changed, which is due to disruption of cell-matrix interaction and increase in metaloproteinase activity. In addition, absence of collagen VI in mouse model results in dysregulation of fibrillogenesis, reduced load and stiffness with an increase in tissue density (Izu *et al.*, 2011a).

Collagen XII as novel disease-causing gene for collagen VI-related myopathy phenotype which is the main focus of our investigation, is a member of FACIT-collagens. Fibril associated collagen interrupted triple helix (FACITs) are a group of collagens which attached on fibril-forming collagens such as collagen I, II and III.FACIT collagens may associate and interact with other glycoproteins and ECM components. The nature of this interaction is not fully understood. FACIT collagens which will be described later are known to be responsible for biomechanical and tensile features of fibril-forming tissues (Young *et al.*, 2002; Zhang *et al.*, 2003).

FACIT-like collagens share the same structural characteristics with FACIT collagens. FACIT-like collagens are collagens XVI, XIX, XXI and XXII. It is believed that these collagens are associated with collagen fibrils mainly in musculoskeletal connective tissues.

However, their distinct function in musculoskeletal tissue is not fully understood yet (Koch *et al.*, 2004).

Among other types of collagens, collagen IV is important due to its unique structure and contribution to basement membrane. It is part of an integrated network which interface between tissues. Its contribution to basement membrane includes many tissues such as neuromuscular tissue, especially near vasculature.

There are several types of basement membranes whose composition is dependent on the anatomical sites. Likewise, collagen IV which is encoded by six different genes has various heterotypes. Collagen IV is known to be an important binding partner for collagen XII in different tissues such as tendon, ligament and musculoskeletal tissue (Khoshnoodi *et al.*, 2008).

All collagens have their own specialized function and contribute to tissue higher order assembly. One major determinant of every tissue`s function and structure for example ligaments and tendons is its collagen composition. In tendons collagen I with 90% of composition is the most abundant collagen type. While the other collagens added to tendons` composition diversity and collaborate in fibril assembly and higher order structure. Furthermore, each collagen in its supramolecular structure could work differently, depending on its location and combination of protein complex interactions (Yang *et al.*, 2014).

1.3.2 *Collagen fibril formation and assembly:*

Collagen fibrils' have got various assembly steps. These steps will be described for the fibril-forming collagen. The differences between assembly of these collagens with collagen VI and XII, the main focus of this research, will be described in detail in chapter 3 and 4. Collagens are synthesized as amino acid chains, assembled into heterotrimeric polypeptides in rough endoplasmic reticulum (RER), packed as trimers in Golgi apparatus then secreted to ECM (Halper and Kjaer, 2014). Fibril-forming collagens (I, II, III, V, IX, XXIV and XXVII) are secreted to ECM as procollagen. Procollagens contain a non-collagenous N-terminal and a C-terminal part which consist of collagenous and non-collagenous domain. In the form of procollagen, collagens cannot assemble into microfibrils. Various enzymes cleave procollagens from C-terminus to N-terminus and make propeptides from procollagens. After propeptide preparation, one large central triple

helical domain with a short collagenous domain will remain. The remaining molecule is called a telopeptide. In most fibril-forming collagens like type I and II, a telopeptide could process to fibril formation, while some collagens, such as collagen III and XI, cannot proceed to microfibrils with retention of C-terminus or partially retained N-terminal. Collagen molecule self-assembly is terminated in striated heterotypic microfibrils with periodicity of 67 nanometres (nm).These microfibrils then proceed into hierarchical assembly process(Halper, 2014a). Figure 1.12 shows the steps of collagens higher structural formation.

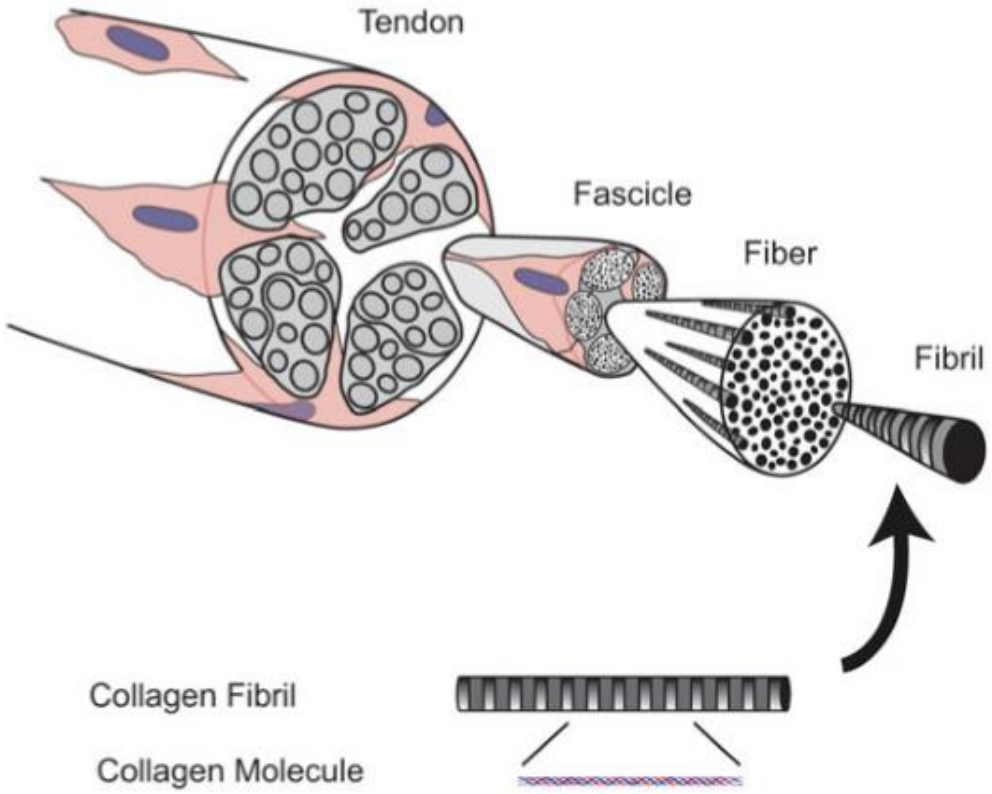


Figure 1.12.Collagens` higher structural formation. Triple helical collagen molecules assemble into collagen fibrils. Collagen fibrils bundled to form fibres. Collagen fibres together with tenocyte within tendons form a fascicle. Each fascicle is surrounded by a loose, connective tissue named endotendon (Mienaltowski and Birk, 2014b).

Collagen fibril structure is influenced by post translational modifications of collagen α chain and covalent modifications which occur after polypeptide chain synthesis. These modifications start during α chain translation(co-translational) and continue during

secretion and assembly. Collagen post-translational modifications include hydroxylation and glycosylation. The number of hydroxyprolin and lysin residues in the collagen amino acid chain is a factor that determinethe amount of hydroxylation and glycosylation. Furthermore, these modifications could influence collagen fibril circumferences (Mienaltowski and Birk, 2014b).

Collagen fibril assembly can decrease by level of enzyme activity, certain mutations in amino acid chains and amount of interfibrilar water. In an *in vitro* experiment of collagen fibrilogenesis, number and diameter of fibrils could affected by pH and environmental temperature (Shoulders and Raines, 2009a).

Collagen fibril assembly starts in deep recesses or channels in fibroblast surface. Collagen fibrils evolved from protofibrils which are small and thin microfibrils. Compartmentalization which occurs in the extracellular matrix under firoblast`s control is responsible for formation of collagen fibrils from protofibrils. While fibrils are large with diameter range between 20-500 nm, the protofibrils are 20-40 nm in diameter. Fibrils are stabilized in the ECM by other matrix molecules such as FACITs and small leucin-rich proteoglycans (SLRPs).

The fibrils then proceed into fibers which could be very large in various tissues. Development of fibres depend on lengh and diameter increase. Fiber length will increase through fibril end overlaps and diameter enlargement requires attachment of fibril to eachother laterally. For diameter increase, all stabilizing molecules must be removed from fibril surface.Formation of further structures from fibers like fasicles and tendons need more complex tissue formation process and depend less on collagen assembly.

As mentioned above collagen fibrillogenesis is controlled by different components such as FACITs and glycoproteins. Collagen fibril assembly controling elements are working as:nucleators, regulators and organizers (Mienaltowski and Birk, 2014b).

During fibre formation a significant increase will occur in fiber diameter. This diameter also controls mechanical properties of fibrils.Collagen V is defined as one nucleator formation element, especially in tendons. Collagen V nucleation provides a mechanism for fibroblast to identfy the fibril site formation. The more nucleation happens, the smaller the diameter of fibrils will be (Mienaltowski and Birk, 2014a). For example, in cornea in which 10% to 20% of fibrils are collagen V fibrils, smaller fiber diameter is observed. Furthermore, cellular organellessuch as cytoskeleton, cell membrane and vesicles are important in nucleation. These organelles contributes in cell-direction mechanisms. This mechanism

helps the fibrils to secrete in cell-defined extracellular domains. This mechanism contributes to cell specific fibrillogenesis and evacuates nucleation site for new round of fibril nucleation. Organizing molecules such as fibronectins and integrins are involved in cell-directed fibril assembly. Integrin assembles fibronectin to collagen fibrils. Furthermore, fibronectin can mediate interactions between fibrils and cells. Fibronectin-collagen fibril network has many sites for fibril assembly. Inhibition of fibril assembly is possible through filling these sites. Furthermore, interventions in fibronectin-integrin interactions or modification of interactions between fibronectin and integrin with other molecules could affect fibril assembly (Kadler *et al.*, 2008).

Once fibrils are preformed, it is required to increase fibrils diameter and length in order to contribute to tissue specific structure. This step is regulated by several macromolecules, among them FACIT collagens and small luecin-rich proteins (SLRPs) are the most important molecules. Both groups have their owntissue specific pattern of expression and both are fibril associated. Various expression patterns lead to different functions and fibril assembly among tissues for these molecules. SLRPsmainly contribute in linear and lateral fibril growth. Class I of SLRPs (decorin and biglycan) and class II (fibromodulin and lumican) are expressed in tendons(Kadler *et al.*, 2008; Mienaltowski *et al.*, 2008). As mentioned before, mouse decorin deficient model or biglycan shows the importance of these molecules in fibril assembly. Deficient mice demonstrate less strenght and load bearing capacity as well as tendon dysfunction.SLRPs function not only during fibrillogenesis but also during injuries, muscle and tendon regeneration and regulate and maintain fibril structure (Halper, 2014a).

FACIT collagens are another regulatory component of extracellular matrix. Like SLRPs, these molecules have a tissue-specific and spatially variable pattern of expression. Fibril growth in cartilage is regulated by collagen IX, while collagen XIV is important in tendon fibril growth. *COL14A* deficient mouse model presents large diameter fibrils in early stages of tendon develoment. Similarly,absence of collagen XII during *in vitro* fibrillogenesis result in large diameter fibrils. As described above, diameter growth is controled by separation of regulatory elements from fibrils and lateral attachment of fibrils into protofibrils. Regarding these findings, some authors suggested that FACITs may act as gate keepers which control and regulate transition of protofibrils into fibrils. Collagen XIV may stabilize collagen fibrils for a period to prevent excessive lateral growth and diameter increase in collagen fibrils (Halper, 2014a).

Structure of FACIT collagens which will be described later, indicates that they might have a role in fibril packing. Through their role in fibril packing they could regulate collagen fibrils diameter (Shoulders and Raines, 2009a).

As indicated above, collagen fibrils assembly is a complicated process which involves several molecules. Mutations in collagen itself and other ECM components can affect this process. However, finding the subsequent result of collagen mutations requires more research on ECM structure. Thus,novel methods for investigating ECM in an *in vitro* and *in vivo* system are required. One challenge to investigate collagen mutations is to distinguish disease-causing variants from polymorphism, as structural variability and unknown role of many collagens makes it difficult to predict about their variant pathogeneicity. In the next section roles of other micromolecules in ECM will be described (Shoulders and Raines, 2009b).

1.3.3 *Other extracellular matrix basic components:*

Collagens as the most abundant and important components of ECM were described in the previous section. Here, we review other components of ECM, mostly from glycoprotein group. These proteins in lower quantity are contributing to the extracellular matrix structure and function. Apart from their maintenance, regulatory and structural role, these proteins are important in ECM disorders directly or indirectly. Fibronectin, laminin and elastin are three examples of these proteins (Kjaer, 2004; Halper and Kjaer, 2014).

1.3.3.1 ***Fibronectin:*** is multidomain structural protein of ECM which act as a master organizer in collagen fibril assembly. Fibronectin interacts with other ECM macromolecules and forms a bridging network which includes collagen fibrils and integrin.Fibronectin has a molecular weight of 230-270 kilodalton (KD) and it is widely distributed in ECM structure of different tissues. Fibronectin is composed of three types of fibronectin (FN) domain: FN I, FN II and FN III. Fibronectin type I and fibronectin type II are stabilizing the molecule through two sulfide bonds in each molecule.Type III fibronectin domain is composed of seven β-barel structure without disulfide bonds. Each FN domain is involved in assembly and ligand-binding of several molecules such as collagen, heparin, fibronectin and elastin. However, fibronectin type III domain which is widely found in collagen molecule is crucial for interaction with collagen fibrils and integrin (Kjaer, 2004).

Fibronectin has twenty isoforms in human and more than twelve isoforms in rodents. An inactive dimer of disulfide bonded fibronectin is secreted to ECM and is activated by integrin interaction. According to its general distribution in tissues, fibronectin is widely

expressed in human body but its expression during morphogenesis, inflamation and cell migration is higher. In tumor cells, the expression of fibronectin is lower but tissues undergoing repair express high amount of fibronectin. Fibronectin has crucial role in ECM assembly. It initiates ECM assembly by its binding to α5β1 integrin receptor (Halper, 2014b). This binding induces fibronectin self-assembly through its N-terminus and lead to cytoskeleton formation and cell contractility. These changes open new binding sites for collagen fibrils on cell surface and accelerate assembly of fibrils into stable but insoluble form. After formation of fibronectin/collagen network, fibronectin involves other molecules assembly to complete ECM formation. Fibronectin has a crucial role in fibril assembly, its unique value for ECM assembly enabled through its capacity to bind to cell surface and several other molecules simultaneously. The multidomain structure of fibronectin is resposible for its binding capacity. By binding to several molecules, fibronectin can assemble collagens such type I and III, proteoglycans and other micromolecules such as heparin. Fibronectin is also a marker of ECM for immunohistochemical studies because of its high expression and wide distribution (Halper and Kjaer, 2014).

1.3.3.2 ***Laminin:*** is large heterotrimeric glycoprotein with 500-800 KD molecular weight. A large variable range of molecular weight is due to different chain size. Each Laminin molecule consists of three chains: α, β and ɣ. These chains also can vary in size, with 200-400 KD for α and 120-200 for β and ɣ. Laminin is located in the basement membrane. Laminin has sixteen isoforms which are encoded by several distinct genes. Laminin has a tissue-specific pattern of expression. Different isoforms of Laminin are expressing in connective tissue of cornea, bone, tendon and ligaments.

Laminin is composed of tandem repeat domains with similarities. Structurally, laminin has two or three short arms with one long arm and it is a cross or T-shaped molecule. Post-translational modification of laminin involves proteolysis of the N-terminus. Laminin binds to integrin, dystroglycan and sulfated proteoglycan via G receptor of its α chain. Furthermore,laminin can adhere to sulfatides and via this, bind to cell surface by its N-terminal receptor (Kjaer, 2004).

Laminin contributes to ECM structure, it can also affect stability, proliferation and migration of associated cells in ECM. Laminin even influence interstitial stroma of ECM. This contributaion occurs through Laminin`s ability to adhere to cell receptors and other moleules from its both N and C terminals. Integrin is an important laminin binding partner in ECM which mainly adhere to Laminin through its α chains (Halper and Kjaer, 2014).

Laminin`s different roles regarding its adherent cells mediated via a complex system which affects chromatin remodeling of promoters and transcription by binding to cell membrane. Like fibronectin-collagen network, laminin cites also form a laminin-collagen VI network in basement membrane. The distinct role of this network is unknown but it helps to transmit forces in musculoskeletal tissue. In addition, investigation in mouse deficient in collagen IV has proven the role of laminin in tendon repair and wound healing. In this deficient mouse collagen IV was absent from outer layer of basement membrane (Taylor *et al.*, 2011). Laminin also has a signaling role to prevent cell apoptosis. However, the details of laminin interaction and different roles remain to be understood.

Laminin is one of the first ECM structures which develop during embryogenesis and it is found to be important for ECM formation in stem cell culture. Furthermore, laminin is a crucial element in different tissues such as blood vessels, nerves, skin, muscle and tendons.

Unfortunately lack of commercial isoforms of laminin makes it a challenge for decipher its complex role in ECM formation and stability. New applications for studying laminin in an *in vitro* system may help to open new insight in its role in different tissues such as muscle (Shoulders *et al.*, 2009).

1.3.3.3 *Tenascins:* are a large family of glycoproteins with molecular weight between 150-380 KD and multimeric structure. Tenascins are given alphabetic names and classified as C, R, W, X and Y based on tissue expression pattern and domain organization. Tenascins are built of various protein domains such as fibronectin type III domains, EGF-like domains, heptad domains and a C-terminus globular domain. Tenascins have tissue-specific development pattern with tenascin-C and -W mostly seen in developing tissues. Tenascin-X and –Y are widley expressed in musculoskeletal connective tissue and Tenascin-R`s expression is predominates in nervous system during development. Among these proteins tenascin-C has been much investigated as it is known tobe involved in tumor development (Tucker *et al.*, 2006).

The role of tenascin-X and Y is more related to this research. Tenascin-X is less glycosylated compared to other tenscins. Its highest expression level is in skeletal muscle. However, it has been found in other tissues such as heart, blood vessels, nervous system and tendons. In musculoskeletal system it has a proven role. It is known to be overexpressed during acute mechanical loading in muscle and muscle stress. In addition,

it contributes to regeneration and recovery of muscle tissue by possessing adhesive effect and play role in tissue coordination (Halper and Kjaer, 2014).

In ECM of muscle, tenascin is part of collagen proteoglycan macromolecules which interact with Collagen IV, FASCITs and other proteoglycans. Dysregulation of this from of tenascin is reported in diseases involving skeletal muscle, skin and connective tissue such as Ehlers-Danlos syndrome (Halper and Kjaer, 2014).

Furthermore, tenascin`s role in musculo-tendinus junction is crucial. In this part tenascin could induce regeneration and wound-healing by presenting de-adhessive effect as well as coordinating tissue regeneration. Some studies suggest that tenascin could orchestrate muscle buil up and repair. Despite its proven role in mechanical loading for tissue and ECM, there are still areas that need to be investigated (Halper and Kjaer, 2014).

1.4 Collagen XII structure and function:

As collagen XII is the main theme of investigation in this research, Its structure and functional characteristics will be discussed in details.

1.4.1 Collagen XII structure and its binding partners:

Type XII collagen as a member of FACIT collagen was discovered in 1989 by Gordon et al (Gordon *et al.*, 1987). They isolated and sequenced a chick embryonic cDNA clone. This clone represented a mRNA and delineated three regions of α1polypeptide chain and one carboxyl domain: non-triple helical carboxyl domain (NC) and a triple helical domain or collagenous domain (C1) (Gordon *et al.*, 1989). The amino acid sequencing of collagen XII cDNA demonstrated that the collagenous domain of collagen XII is similar to collagenous domain of collagen IX. This evidence has led to categorizing two molecules in one category. Collagen XV is another member of this group but collagen XII is the largest member of FACITs (Dublet *et al.*, 1989). Further investigation revealed collagen XII consists of three non-collagenous domain (NC) and one collagenous tail. The non-collagenous domains consist of several fibronectin type III (FNIII) regions and some von wilbrand A domains (Koch *et al.*, 1992). The collagenous part comprise a small percent of molecule, while non-collagenous domains specially NC3 is extensive and help to relate collagen XII to other molecule such as Heparin, tenascin-X and von willbrand (Koch *et al.*, 1992).

Collagen XII exist as a homotrimer that assembles from three identical α chains encoded by *COL12A1* genes (Bader *et al.*, 2009).Differential splicing within these domains give rise to a small (220 kD) and large (320 kD) collagen XII subunits. The small subunits does not

have the first NC domain. Only the large subunit can carry glycoseaminoglycans (Koch *et al.*, 1992). Figure 1.13 shows a schematic picture of collagen XII and its variants.

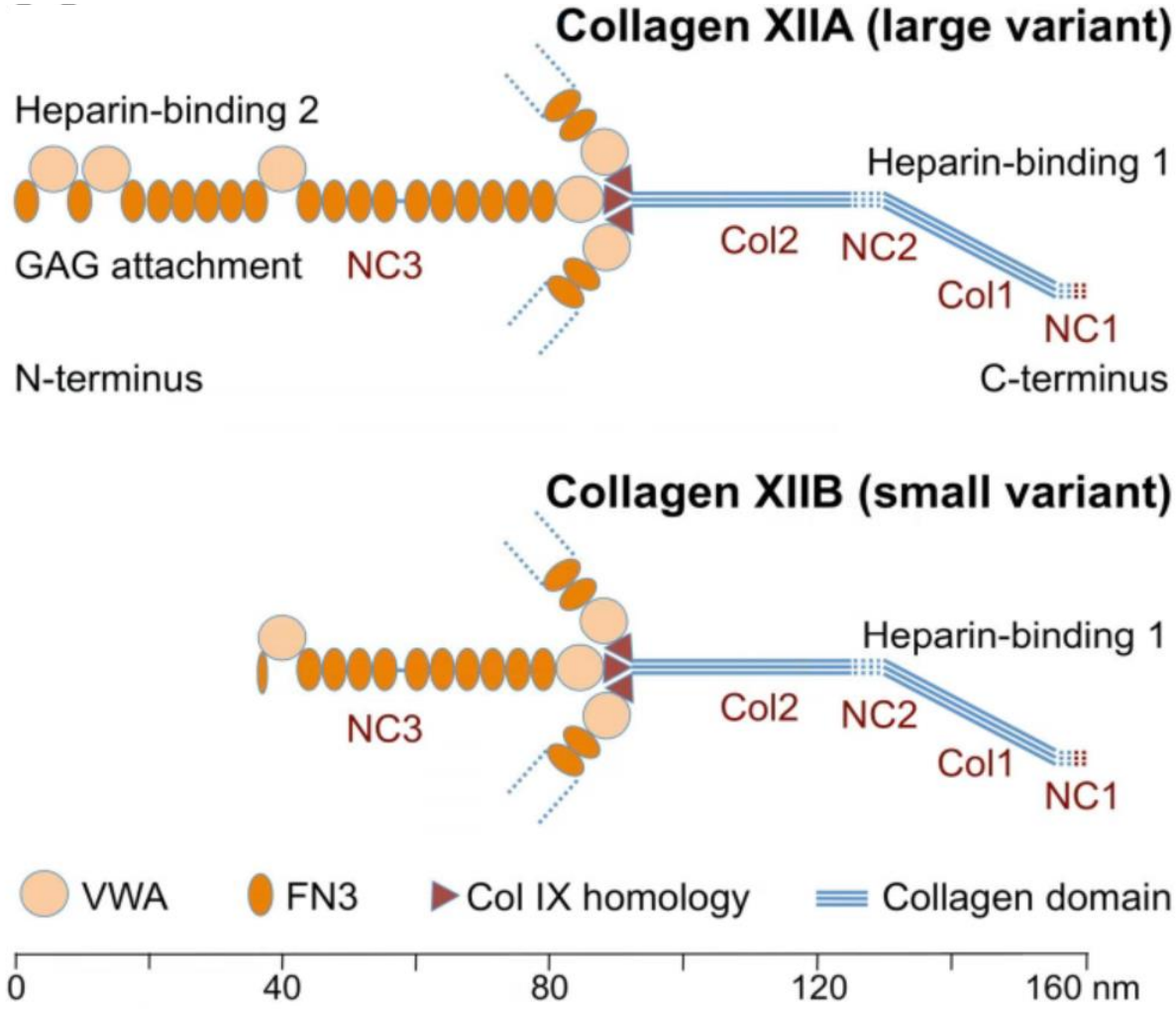


FIGURE 1.13.Collagen XII large and small splicing variant with their molecular regions (Chiquet *et al.*, 2014).

Koch et al generated specific antibodies for alternatively spliced variants to investigate their characteristics (Koch *et al.*, 1995). They reported large variant has a more restricted expression pattern than the small variant in embryonic tissue. The large variant only expressed in feather bud in chick, while the small variant expressed in all dermis part. They showed with their speific antibodies that collagen XII has homo and heterotrimers. The two subunits differs in ligand binding. The small one interacts weakly with heprin but the large subunit with more NC domains has extra heparin binds (Koch *et al.*, 1995). So far, collagen XII structure becomes fascinating entity for this molecule. Its small collagenous part is resposible for collagenous chracteristics while its NC arms helps to

interact with various other molecules such as Decorin, Tenascin-X and collagen I containing fibrils. Like other collagens, understanding of collagen XII role is based on interpretation about its structure (Chiquet *et al.*, 2014). Figure 1.14 demonstrates collagen XII interaction with other molecules in ECM.

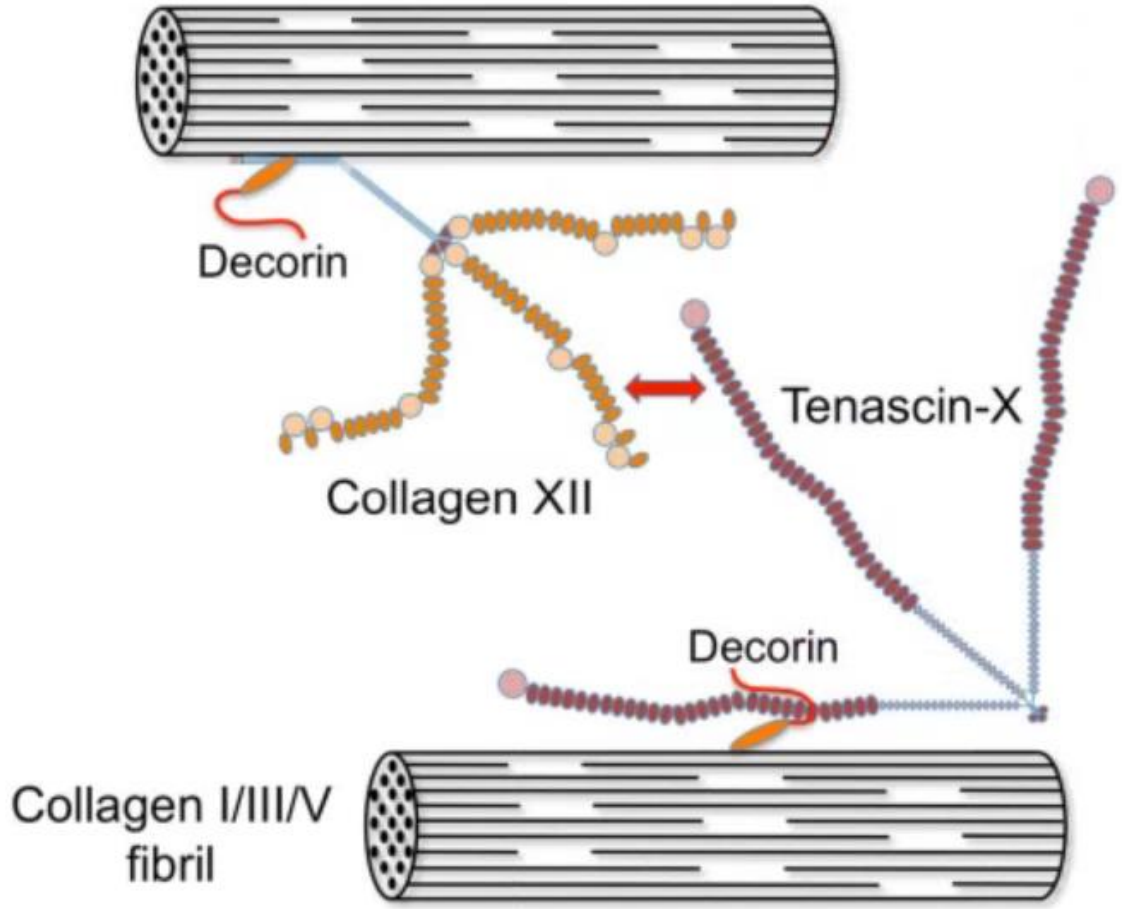


Figure 1.14.Collagen XII cross-bridging with its binding partner in extracellular matrix. Collagen XII cross-bridging with collagen I containing fibrils through its collagenous surface. This might be facilitated by Decorin AND Tenascin-X molecules. The pink arrow shows Fibronectin type III domains in collagen XII and Decorin which facilitated the interaction between two molecules. The link between collagen XII and collagen I containing fibrils occurs through collagenous domain in C-terminal of collagen XII (Chiquet *et al.*, 2014).

1.4.2 Collagen XII function:

So far the function of the two distinct form of collagen XII remains a mystery. Koch at al showedthat biochemically and ultrastructurallycollagen XII can be attached to collagen I fibrils when it is present during fibril formation but not after that. In addition, Removal of the collagenous domain of collagen XII reduces its coprecipitation with collagen I. Removal of collagen XII from *In vitro*environment for collagen fibrillogenesis could lead to increase in

collagen fibril diameter. They also indicated that collagen XII is specifically associated with fibrillar collagens like collagen I (Koch *et al.*, 1995). Thus, collagen XII could play a role in in collagen I contaibibg fibril formation and diameter (Koch *et al.*, 1995). As previuosly showed, collagen XII is attached to collagen I contaning fibrils through its collagenous domain, while its non-collagenous arms facilitating its interaction with Decorin,Tenascin-X and Heparin. Collagen XII and its non-collagenous binding partner could form a flexible bridge between ollagenous fibrils. *COL12A1* knock out mice study showed phenotype of muscle weakness and increased elasticity of muscle-tendon units (Zou *et al.*, 2014). These data suggest collagen XII network might work as absorber of sheering stress between collagen fibrils (Chiquet *et al.*, 2014; Zou *et al.*, 2014).

COL12A1 knockout mice also demonstrating skletal deformities as short bones and spinal kyphosis.Histological findings showed disorganized collagen networks in bone and decrease in bone specific proteins (Osteocalcin and Osteopenin). In addition Osteocytes of *COL12A1* knockout mice shows less dendritic processes and ecreased polarity (Zou *et al.*, 2014). These data suggest that collagen XII is important for bone formation and structure. However, it is not clear wether collagen XII role mediated through direct interaction with cell receptors or other molecules (Izu *et al.*, 2011b).

In summary, The previous findings suggest a role for collagen XII in connective tissue patholog. In addition, they established a link between the structure and binding partner of this FACIT molecules and its function as a regulator of collagen fibrils formation and load bearing element (Koch *et al.*, 1995; Chiquet *et al.*, 2014).

1.5 Overview:

In the next chapters we aim to describe novel gene from collagen family, Collagen XII, which we found as disease-causing in affected individuals with phenotypes overlapping musculoskletal as well as connective tissue disorders. These patients were classified as Bethlem phenotype before. In the first result chapter we aim to identify the novel disease-causing gene in a cohort of Bethlem-like myopathy patients with no mutations in *COL6A1-A3* genes. These novel identified gene then evaluated with immunofluorescence and immunoblotting techniques among normal and mutant individuals. In this step, as we describe later we could identify *COL12A1* as a disease-causing gene in three families of Bethlem-like myopathy patients with no *COL6A1-A3* mutations. The further evaluation reveals decrease in collagen XII signals in the affected individuals.

In the second result chapter, we aimed to investigate collagen fibers structure from *COL12A1* mutant individulas and compare it to healthy donors.In addition collagen fibrils diameter from *COL12A1* mutant individual and healthy donors were measured after culturing fibroblasts of affected individuals and controls. In addition, collagen molecules were extracted from cultured fibroblasts and after inducing fibrillogenesis, the diameter of fibrils were measured. These experiments reveal that there are no differences in fibril diamters between control and affected individuals.

In the third result chapter we aimed to calculate C2C12 differentiation index, when cocultured with fibroblasts of *COL12A1* affected individuals and healthy donors.in direct contact and inserted cell culture dish (without direct contact).

These experiments reveals that direct contact between fibroblasts and C2C12 could decrease C2C12 differentiation index in *COL12A1* mutant cells compare to controls. Overall, our findings suggest a role for *COL12A1* gene in collagen VI-related myopathy patient with no mutations in *COL6A1-A3* genes. However, more investigation is needed to decipher the exact *COL12A1* mutant patinets pathomechanism.

Chapter 2 Materials and Methods

2.1 Primer design:

The program Primer3 Plus was used for designing all primers (http://frodo.wi.mit.edu/primer3). For each primer the standard length was set as minimum: 19 nucleotides, maximum: 22 nucleotides and the optimal size was 21 nucleotides. The Eurofins MWG Operon website was used to synthesize the primers. The long mRNA transcript for *COL12A,* which is NM-004377.5, was used to design primers for the *COL12A1*gene. *COL12A1* was sequenced in twelve amplicons in cDNA level. To sequence single polymorphisms in genomic DNA the above program were used. Appendixes A and B and C show the sequence, lengths, and Temperatures (TM) for each primer in this study.

2.2 Polymerase chain reaction experiment:

Two different Polymerase chain reaction (PCR) programs were used to amplify DNA fragments. From the two programs described below for PCR, the first one was used for DNA amplification of less than 1000 kilo base (KB) and the second one was used for longer DNA amplicons. Annealing temperature in step two and three in the first program varied from 56 to 60 degrees Celsius (°C) according to the suggested annealing temperature by Eurofins for primers. In the second program the two annealing temperatures were constant. Table 2.1 shows materials and their concentration in each reaction. Table 2.2 describes the set-ups of two PCR programs.

Material Name	Company Name/Catalogue number	Amount of reagent used in 50 microliter (µl) master mix	Amount of reagent used in 25 microliter (µl) master mix
PCR Buffer	Molzym-F18v32P013	5 µl	2.5 µl
PCR Enhancer	Molzym-119102030v353	4 µl	2 µl
DNTPs (dATP, dTTP, dGTP, dCTP)	Fermentas/R0141, R0171, R0151, R0161)	2 µl	1 µl
Moltaq polymerase	Molzym-C07V462P010	1 µl	1 µl
Patient DNA	Not applicable	1 µl	1 µl
Forward and backward primers (Eurofins)	Eurofins, MWG operon	1 µl	1 µl

Table 2.1. Materials used for the PCR experiments.

Program one				Program two		
Stages	Temperature	Minutes	Cycle	Temperature	Minutes	Cycle
1	94°C	02:00	1	94°C	02:00	1
2	94°C	00:15-01:00	40	94°C	00:10	9
3	55-60°C	00:15-02:00	40	55°C (annealing temperature)	00:30	9
4	72°C	01:00-02:00	40	68°C (annealing temperature)	02:00	9
5	72°C	07:00	1	94°C	10:00	19
6	-	-	-	55°C	00:30	19
7	-	-	-	68°C	02:00	19

Table 2.2.Two different PCR set-ups.

The 2% w/v agarose (Sigma-Aldrich, A9539) gel stained with 1 µl SafeView (NBS Biologicals ,NBS-AG 500) was used to visualize PCR products of less than 1000 KB and 1% w/v agarose gel was used for products of more than 1000 kb. The running time for each gel was at least thirty minutes at 80 Volts (V) in an electrophoresis tank (Bio-Rad). Visualisation of gels under UV light and documentation was done using a GelDoc-IT system.

2.3 DNA purification and sequencing:

Two methods were used for purifying PCR products: one for purifying from the gel after electrophoresis and the second to purify from the PCR reaction directly. QIAGEN

protocols (28706-Gel purification and 28106-PCR purification) were used to purify the DNA.

2.4 *In silico* analysis tools:

The Bioedit program (http://www.mbio.ncsu.edu/bioedit/bioedit.html) was used to align sequences with the reference sequence. The mutation surveyor program (http://www.softgenetics.com/mutationSurveyor.html) was used to identify point mutations in cDNA amplified segments. Mutation taster (http://www.neurocore.charite.de/MutationTaste) and polyphen (http://www.genetics.bwh.harvard.edu/pph2) databases were used for variant analysis and predicted pathogenicity. 1000 genomes databases (www.internationalgenome.org) and the NHLBI Exome Sequencing project (EVS) (evs.gs.washington.edu) were used to differentiate polymorphisms from rare pathogenic variants.

2.5 Genomic DNA extraction:

MagAttract DNA blood Mini M48 kit (Qiagen, 951336) was used to extract genomic DNA from blood samples of candidates for exome sequencing by the Northern Genetics Service in Newcastle. The DNA easy blood and tissue kit (Qiegen, 60504) was used to extract genomic DNA from the fibroblasts of individuals who required confirmation of existence of pathogenic variant for BM2 and BM5 families. For BM1`Whole exome sequencing (WES) was performed at de CODE genetics (Iceland) in 30 individuals (20 affected, 10 unaffected) from 9 families. Illumina Nextera Rapid Capture exome kit (37 Mb) was used and sequenced paired-end on Illumina HiSeq2000 sequencer. Burrows Wheel Aligner was used for variant calling, sequence aligner and variant detection

2.6 Exome sequencing analysis:

To prepare template DNA for exome sequencing in five Bethlem-like patients, adaptive focused acoustics (Covaris, Illumina Paired-End Sample Preparation kit) was used to produce the DNA fragments of 150 base pair (BP). Agilent Sure Select Target Enrichment System and Sure Select Human All Exon Kit v2 were used for the Paired-End Sequencing Library (v2.0.1) of Illumina. Fragments were sequenced with 75-bp paired-end reads output by an Illumina genome analyzer. Alternatively, the TruSeq system from Eurofins was used for size-selection and to purify the coding region before sequencing DNA

fragments on the Illumina platform. Consensus genome (hg19) was used to align the raw sequencing read. The result consequently was sorted and converted to a BAM file by using Mosaik [version 1.1.21; http://bioinformatics.bc.edu/marthlab/Mosaik (9 May 2012, date last accessed)]. Variant calling processes were done by the SAMTOOL method. Dindel was used to optimize indel calling. The final list of variants was evaluated by the Genome UCSC browser (https||:genome.ucsu.edu). Other *in silico* analysis systems were used to evaluate variants predicted to be pathogenic. For BM1´,WES in 30 individuals (20 affected, 10 unaffected) from 9 families was performed at de CODE genetics (Iceland) using Illumina Nextera Rapid Capture exome kit (37 Mb) and sequenced 90 nt paired-end on Illumina HiSeq2000 sequencer. Sequence alignment, variant calling and functional annotation were performed with Burrows Wheel Aligner, Genomes Analysis Tool Kit and Variant Effect Predictor (VEP).

2.7 Cell culture and Immunofluorescence staining in fibroblasts:

Primary dermal fibroblasts provided by the CNMD Biobank, Institute of Genetic Medicine, Newcastle University, and Normal Human Dermal Fibroblasts (NHDFC) (Promocell, 106k-05a) were used as controls and cultured under aseptic conditions. Medium change, splitting and cell freezing were done every two days during the experiment.

Material Name	Company/Catalogue Number	Initial Concentration
Dulbecco's Modified Eagle Media(DMEM)	Gibco-41966-029	4.5 g/l(D-Glucose, Glutamate, Pyruvate)
Trypsin	Gibco-25300-054	0.05%-250 ml
Phosphate Buffer Saline (PBS)	Gibco-20012-019	
Recovery Freezing Media Fotal Bovine Serum	Gibco-12648-010 Gibco- 10064	

Table 2.3 shows the materials and amounts used for cell culture.

According to the immunofluorescence staining protocol, cell lines were seeded into 8-well glass chamber-slides (Thermo Fisher Scientific, 154534) at 90% confluency. On the second day, filtered sterilised ascorbic acid (Sigma-Aldrich, 4544) was added to the media at 1:20 dilution. Staining was done on the fifth day after seeding the cells. Before staining cells were checked under the light microscope to make sure they had appropriate confluency. Cells were fixed at room temperature for 15 minutes in 500µl per chamber of 4% paraformaldehyde (Sigma-Aldrich, 158127) in PBS. After washing with PBS, 100 µl blocking reagent with and without Triton (Sigma-Aldrich,T878) was added to appropriate fields in order to block nonspecific binding antibodies and permeabilize the membrane. Blocking reagents were made as follows: 5mililitre (ml) Foetal Calf Serum (Gibco,10437036) and 5 ml 10 X PBS in 40 ml distilled water, for blocking reagent with triton; 50 µl Triton X-100 (Sigma-Aldrich, T8787) was added. After incubating for one hour at room temperature and washing with PBS, 100 µl dilutions of corresponding primary antibodies were added to appropriate fields (PBS was substituted in the negative controls chambers) and incubated for one hour. After 3x20-minute washes with PBS, 100 µl secondary antibodies, goat anti-mouse Alexa fluor-488 and goat anti-rabbit Alexa fluor-594 diluted 1:1000 µl in a blocking buffer, were added. Incubation and washing were repeated three times. Finally, the slides were mounted in Vectashield mounting medium containing

6-diamidino-2-phenylindole (DAPI; Vector Laboratories, H-1200). All slides were visualised on a Zeiss Axioimager microscope using 20 and 40 objectives, with Axiovision 4.5 software. Table 2.4 shows the concentration and features of primary and secondary antibodies in this study.

Antibody name	Product Description	Company/Product Code	Initial concentration	Used Concentration
Anti-collagen VI antibody	Polyclonal rabbit	ABCAM/ab3062	1 mg/ml	1/100 µl
Anti-collagen XII antibody	Polyclonal rabbit	Provided by prof Manuel Koch, Cologne University	1 mg/ml	1/1000 µl
Alexa 594 Anti-rabbit	Polyclonal donkey	ABCAM/A11012	2 mg/ml	1/1000 µl
Alexa 488 Anti-goat	Polyclonal donkey	ABCAM/A11002	2 mg/ml	1/1000 µl

Table 2.4.Features of primary and secondary antibody in this work.

To optimize immunofluorescence staining, some additional steps were added to the conventional protocol. On the staining day, before blocking non-specific antibody stage, cells were incubated with 0.05 NaCl/HCl for 15 minutes. This step helped to permeabilize cell membranes for primary antibodies.

2.8 RNA Extraction and conversion to cDNA:

Dermal fibroblasts were cultured to 90-100% confluency in DMEM medium with 10% FBS. Cells were trypsinized, centrifuged and kept in PBS. RNA was extracted according to the RNeasy Mini-Kit (Qiagen, 74104). 18 µl of mastermix containing RNA were incubated for 2 minutes at 42°C. Then 2 µl Superscript reverse transcriptase III (Invitrogen, 18080-44) was added and heated at 42°C for 50 minutes and 70°C for 15 minutes.

2.9 Muscle biopsy staining:

Collagen XII primary and secondary antibodies were used at the same concentration as the cell immunofluorescence staining. On the first day, after warming up the slides for 1 hour, they were placed in a Hellendhal jar and washed with 0.1% Triton X100 in 1xPBS for 1 hour. Then protein block (Dako, 201402) was added to cover the whole slide surface and after 2 minutes was replaced with primary antibodies which were diluted in protein blocks. Slides were stained overnight at 4°C and remained on the shaker. On the second day, the secondary antibodies were added and slides were incubated at room temperature for 90 minutes. Then antibodies were washed with PBS and slides placed in a Hellendhal jar for a 30 minute wash with PBS on the shaker. Finally, slides were allowed to dry and DAPI with Vectashield was added. Images were visualized with the Axioimager microscope. Medium (DMEM) with 10% foetal bovine serum was used to feed cells every two days. The cells

2.10 Fibroblast Transfection:

The Normal Human Dermal fibroblast cells, NHDFC (Promocell, 106k-05a), passage number<3 were grown on T75 flasks (Sigma-Aldrich, Z707511) till they reached 80% confluency. Dulbecco's modified eagle were cultured in T75 flasks for 6 days before transfection. Amaxa Human Dermal Fibroblast Nucleofector Kit (Lonza, CC-2511) was used to transfect cells. At 80% confluency, media were removed and cells were washed with 10 ml HBSS (Lonza, 04-315Q). 5 ml trypsin was added and cells were incubated for 2 minutes at 37°C. Detachment of cells was checked with the light microscope (Leica). Then trypsin was neutralized with TNS (Lonza, CC5002) with the equal amount of 5 ml. 100 µl of trypsin+ TNS media were collected and cells were counted with a hemocytometer (Sigma-Aldrich). Relative amount of media, 6 ml, were collected in order to have 5 $x10^6$cells for nucleofection. The media were centrifuged (top table Eppendorf centrifuge, 5417R) at 200 G for 10 minutes and cell pellets were resuspended in 100 µl nucleofector kit solution. 10 µl of *COL12A1* shRNA (Santa Cruz, sc72958SH) or 5 µl of Green fluorescent protein GFP (Thermo Fisher Scientific), provided with the kit were added to each sample. Samples were transferred into provided cuvettes and program 0U22 was used for transfection in the electroporation machine (Lonza). This program used electric shock which is adjusted with chemical solution to move in recombinant products.

Samples were then seeded on 6 well plates (Sigma-Aldrich, CLS3516) with appropriate labelling for collagen XII and GFP controls. 1.5 ml preincubated media were added to them. Eight hours later puromycine (Invivogene, 10 mg/ml, 58-58-2) were added to samples in order to select transfected cells. Samples stayed in puromycine for 10 days and media was changed every day. The surviving cells were collected into 96 well plates (Sigma-Aldrich, CLS3912) and left to grow in 100% confluency. Western blotting and RT-PCR were used to detect gene knock down.

2.11 Immunoblotting:

Fibroblasts were cultured on 6 well plates (Sigma-Aldrich, CLS3516) with DMEM medium, 10% FBS and 50 µg/ml L-ascorbic acid (Sigma-Aldrich,A4403) until they became 100% confluent. Cells were washed with PBS. Then media were replaced with serum-free DMEM with 50 µg/ml ascorbic acid for 48 hours. For preparing medium extract for westernblot, the 24-48 hour media containing ascorbic acid was collected in a 15 ml falcon tube (Sigma-Aldrich, CLS430791) and centrifuged for 5 minutes at 1500 rpm. Then media supernatants were split into 100 µl aliquots. 900 µl ethanol was added to each aliquot. After mixing, samples were frozen at -80ºC for 3 hours or longer before running westernblot.

For preparing cell layer extract, the cell layer was rinsed with cold 1x PBS and 6 well-plates were kept on ice for five minutes. 200 µl complete RIPA buffer consisting of 150 mM sodium chloride (Sigma-Aldrich, S9888), 1 ml Triton X100, 5% sodium deoxycholate (Sigma-Aldrich, 302-95-4), 0.1% sodium dodecyl sulphate, (Sigma-Aldrich, 71727), 50 mM Tris (Sigma-Aldrich, 77-86-1) with pH 8.0 were added to each well. The cell layers were scraped and collected in a 1.5 ml Eppendorf tube (Sigma-Aldrich, T9661). The tubes were put on ice for 5 minutes, then spun down at 1400 rpm for 15 minutes at 4°C. The supernatant and cell lysate were kept separate. For running samples of cell lysate 2 µl of each supernatant were combined with 5 µl 4×LDS loading buffer (ThermoFisherScientific, 84788) containing 10% beta-mercaptoethanol (Sigma-Aldrich, 560000), heated for 5 minutes at95°C.

During the running stage, samples were loaded on to synthetic 1 mm Nupage (Life technologies) gels. 4-12% Bis Tris gels (Life technologies, NP0321BOX) were used for proteins less than 120 Kd as Emerin (Novocastra 6120-07, 1:50) and collagen VI. 3-8%

Tris Acetate gels (Life Technologies, EA0357BOX) were used for proteins of more than 200 Kd such as collagen XII.

Bis Tris gels were run for 45 minutes and Tris Acetate gels were run for two hours at 150 mV in the XCellSureLock system (Invitrogen). For molecules<120 Kd, 35ml Tris acetate running buffer (Thermo Fisher Scientific, LA0041) was washed with 700ml water. For 120Kd<molecules 35 ml of MOPS running buffer (Thermo Fisher Scientific, NP0001) was used. Then mini-gels were cut and put into the transfer buffer. PVDF membranes (Thermo Fisher Scientific, 88518) were pre-wetted in 100% methanol for thirty seconds and the transfer step was done at 350 mA for 1.5 hours in a Bio-Rad mini-Protein III electroblot system. Membranes were blocked with 5% BSA (Sigma–Aldrich) in 1X TBST for 1 h before incubation with primary antibodies; anti-human collagen XII (gift of Prof. Manuel Koch, 1:2000) and anti-Emerin (Novocastra 6120-07, 1:50) overnight. 10x TBST is made up of NaCl (87.66 g/l), 2M Tris (pH 7.8) (250 ml/l), Tween-20 (5 ml/l, Sigma-Aldrich, 9005-64-5), Ultrapure water (to 1 l) and diluted to 10x for the working solution.

The day after, membranes were incubated with secondary antibodies; goat anti-rabbit, HRP conjugated (Invitrogen G21234, 1:3000) and goat anti-mouse, HRP-conjugated (Invitrogen G21040, 1:3000) for one hour. High mark prestained protein ladder (Thermo Fisher Scientific, LC5699) for collagen XII immunoblotting and standard protein ladder (Thermo Fisher Scientific, 26616) for collagen VI were loaded on to gels to differentiate between proteins. Agfa X-ray films (VWR) were used to reprint images of protein bands. Imaging was performed on a Bio-spectrum imaging device (UVP, Upland, CA, USA) using Vision Works v7.0.2. Collagen XII bands are predicted to be found at 330 KD, collagen VI bands at 110 KD and Emerin bands as control at 45 KD. An Image J analyzer was used for statistics and band calculation. Oddyssey image software (Li-cor biosciences) was also used to reprint the images. In order to use the Oddyssey system, following primary staining, membranes were incubated with goat anti-rabbit (1:10000) and goat anti-mouse (1:10000) Li-cor system secondary antibodies. Special Li-cor blocking reagents were used to block primary and secondary antibodies.

2.12 Electron microscopy:

2.12.1 *Transmission electron microscopy for tissue:*

Tissue from skin biopsies of normal and mutant individuals was rinsed in 0.1 M sodium cacodylate (Sigma-Aldrich, 233854) post-fixed in 1% OsO_4 (Agar Scientific) for 1 h and dehydrated in sequential steps of ethanol (25, 50, 75 and 100% twice) prior to impregnation in resin (TAAB Lab Equipment) and polymerized at 4°Cfor 24 h. Semi-thin sections of 1 µm were cut and stained with 1% toluidine blue (Sigma-Aldrich, T3260) in 1% borax. Ultrathin sections of 70 nm were cut using a diamond knife on a Leica EM UC7 ultramicrotome. Sections were mounted on Pioloform -filmed copper grids prior to staining with 1% aqueous uranyl acetate and lead citrate (Leica) and viewed on a Philips CM100 Compustage Transmission Electron Microscope. Images were taken at a magnification of ×20 000 and ×46 000.

2.12.2 *Immunogold microscopy on cultured cell:*

24 well-inserted tissue-culture plates (Cornings, 3470) were used for growing NHDFCs and patient fibroblasts. Cells were grown to full confluence. A DMEM medium with 10% FBS and 100 mg ascorbic acid was used to feed the cells. After five days, the process of fixing for immunogold electron microscopy was started.

In immunoelectron microscopy of adherent cultured cells, cells can be processed while still attached to the base of the plastic culture dishes. We describe a pre-embedding immunoelectron microscopy technique for chemically fixed tissue culture cells attached to the base of a culture dish.

After decanting the tissue culture medium and rinsing the cells with PBS (Gibco, 10014-422), the PBS was aspirated. 3% PFA (Sigma-Aldrich, P6148) in 0.1 M PB (Thermo Fisher Scientific, P5244) was added and gently swirled. Then the cells were kept for 15 minutes at room temperature. The fixative was removed and cells twice again were rinsed with PBS. The washing Buffer then removed and cells were kept on 0.1% Triton X100 (Sigma-Aldrich, T8787) in PBS to permeabilize the cell wall. Incubation was done for 25 minutes at room temperature. Then the blocking reagent was aspirated and cells were rinsed twice with PBS. Cells were washed again for 5 minutes three times with 0.02 M glycine (Sigma-Aldrich, 56-40-6) in PBS. The washing buffer was aspirated and 3% BSA (Sigma-Aldrich, 10416-59-8) in PBS was added. The cells were incubated for 30 minutes at room temperature to block the non-specific binding antibodies. Then the blocking buffer was aspirated and the cells were washed with 0.1% BSA in PBS. The primary antibody,

collagen XII anti-rabbit was diluted with 1% BSA in PBS. 1 µl of antibody was added to 500 µl of 1% BSA in PBS. The cells were incubated with the primary antibody for 3 hours at room temperature. Then the antibody was aspirated and cells were washed with 0.1% BSA in PBS. The secondary nanogold antibody, anti-rabbit IgG (Nanoprobes, 12348) was diluted 1 µl in 50 µl of 1% BSA in PBS. The cells were incubated with the secondary antibody overnight in a refrigerator. On the second day, the nanogold solution was aspirated and cells washed six times for five minutes with 0.1% BSA in PBS. Then the cells were washed six more times only with PBS. Following the second wash steps cells were incubated in 1% Glutaraldehyde, GA (TAAB) in 0.1 M PB for 10 minutes at room temperature. After removing the fixative by aspiration, the cells were washed again with PBS for five minutes. Then the cells were incubated four times for 15 minutes with 50 mM HEPES-NaOH pH 5.8. After the last wash with HEPES solution (Sigma-Aldrich, 7365-45-9), the cells were washed twice with distilled water. Then silver-enhanced Nanogold particles (Nanoprobes, 2012) were added. The silver-enhanced solution was prepared as described in the protocol (Nanoprobes, 2012). Solutions A (initiator) and C (activator) were added and followed by solution B (moderator). Then one drop of the enhanced solution was added to wells apart from keeping one row for negative enhancement. The cells were incubated for 6 minutes in a water bath to activate the enhancement particles. The enhancement solution aspirates very quickly and cells were washed five times for 1 minute in distilled water to terminate the silver enhancement reaction. The cells again were washed in PBS once for five minutes. Then the cells were fixed for 1 hour at 4°C in 0.5% Osmiumtetroxide, OsO_4 (TAAB) in 0.1 M PB. After aspirating the post-fixatives the cells were rinsed twice for 5 minutes in 0.1 M PB. Then 0.1 M PB was aspirated.

The following procedure was used to embed attached cells after growth on inserted wells and staining. The cells were incubated for 10 minutes in 50% ethanol to dehydrate them. The dehydration process was followed by a second 10 minutes in 70% ethanol, then 10 minutes in 90% ethanol, the fourth 10 minutes in 95% ethanol and the last part in absolute ethanol for 15 minutes. Ethanol was removed after the last part without allowing the cells to dry. Epoxy resin (Fibre Glass Direct) was filled in each well to make it half full. The specimens were incubated for one hour at room temperature. The epoxy resin was aspirated and new epoxy resin was added to the cells and left overnight. The next day the new epoxy resin was added and allowed to polymerize for 4 days. The fifth day first left the polymerized resin plate to cool in room temperature, then the plastic part of the tissue

culture plate was removed as much as possible with pliers. The goal is to have separated resin blocks each from a well.

Then each of these removed wells was placed with the bottom facing upward. With a hammer, the bottoms of the wells were stroked to detach the epoxy resin together with the resin-embedded cells from the plastic plate. Then the resin block was cut into small pieces with a serali saw, and the samples were cut into pyramid-shaped pieces. The ultrathin sections were then placed in var-coated TEM grids. These grids were coated with metal followed by carbon backing up to inhibit negative charges and thermos damage. The sections were then stained with 2% aqueous uranyl acetate (TAAB) solution for 10 minutes. The grids were washed with distilled water.

The sections were first checked with light microscopy to make sure that we had got good samples. Then electron microscopy was used to get images. Image J analysis software was used to analyse the images.

2.13 *In vitro* fibrillogenesis:

In order to check whether there is any difference between collagen fibril sizes, a method was used to extract collagens from mutant and normal dermal fibroblasts, then the molecules were induced for *in vitro* fibrillogenesis. For extracting type I collagens, NHDFC and mutant cells were grown to full confluency in six T75 flasks (Fisher Scientific, 35135). The fibroblasts were fed with Dulbecco's modified Eagle medium, DMEM which has the pH of 7.4 and buffered with 24 mM sodium bicarbonate (Sigma-Aldrich, 144-55-8) and 25 mM HEPES. 10% foetal bovine serum (Gibco, 10500-056) was added to the medium. The cells were incubated at 37°C, with 5% carbon dioxide and 95% oxygen supply. Once the cell cultures were confluent, the growth media were removed and cells were incubated at 37°C in 0.5% calf serum in a DMEM medium. 100 µg/ml streptomycin sulphate (Sigma-Aldrich, S6501-5G) and 100 µg/ml penicillin G sodium (Thermo Fisher Scientific, 15140122) was added. The media were supplemented with daily 100 µM ascorbic acid for four days. Each day the media were collected. Following the fourth day, the cells were incubated with DMEM which has 10% foetal bovine serum and allowed for three days for regrowth before repeating the ascorbic acid treatment and collagen harvest. The collected media were precipitated each day with 2 volumes of 95% ethanol to 1 volume of media. An amount of 20 µl of 200 Mm Phenyl methansulfonylfluoride (PMSF) (Sigma-Aldrich, P7626) with a final concentration equal to 4.0 Mm was added to each solution for

preventing protease activity. The precipitates were collected by centrifugation at 10000x g for 45 minutes. The supernatant was discarded and the pellet re-suspended in 0.5 normal acetic acid (Sigma-Aldrich, 537020) and kept overnight. The day after, the solutions were recentrifuged at 10000x g for 30 minutes and digested with 1 mg/ml pepsin. The supernatant was kept for further use. The soluble protein was dialyzed overnight at 400-fold volume excess of 0.5 N acetic acid and digested with 1.0 mg/ml pepsin (Sigma-Aldrich, P4656) on ice overnight. These samples were lyophilized and then dissolved in 5 mM acetic acid to a final concentration of 1.0 mg/ml. The concentration of samples was checked with a modified Bio-Rad protein assay. The samples were then kept on -80 freezers and were used for collagen fibrillogenesis.

2.14 Collagen fibrillogenesis assay:

The concentrated and extracted protein with 1 mg/ml concentration from previous experiments was used to induce fibrillogenesis. One control sample of collagen I (abcam, AB34710) extracted from a rat tail was used as a control for the fibrillogenesis experiment. 1 mg/ml of extracted collagen samples from the mutant and controls were dialyzed in 500 ml of 5 mM acetic acid (Sigma-Aldrich, 537020) at room temperature on the shaker to induce fibrillogenesis. Samples stayed on the shaker overnight. The day after, dialysis clips (VWR, 80-6484-32) were opened and 1 ml of solution with induced fibrils were taken to the electron microscopy unit for negative staining. 1 µl of each sample were taken to the electron microscopy unit and diluted with 10 µl of water, then uranyl acetate with 10% dilution was added to samples for 10 seconds and dehydrated under lamps. The samples were then transferred on to the bench and an electron microscopy grid was floated on one droplet of samples. The excess of water was absorbed with absorbent papers. Then collagen fibrils were viewed via electron microscopy.

2.15 Fibroblast / C2C12 coculture assay:

Small plastic flasks were used for growing cells in this assay. Two control fibroblasts, two mutant fibroblast and one Duchenne muscular dystrophy (DMD) samples were used. Each cell line used in this assay had less than 5 passages to begin with. One NHDFC cell line was purchased from biologic company (Promocell, C-12352). The other control fibroblasts were provided by biobank in Newcastle Centre for Neuromuscular Disease.

These fibroblasts were obtained from skin biopsy of one orthopaedic anonymous patient in Royal Victoria Infirmary (RVI) hospital affiliated to national health system (NHS). One DMD fibroblast which were used as a positive control was provided by the biobank. These fibroblasts were obtained from a skin biopsy of a confirmed DMD patient with deletion in exon 49 and 50 of DMD gene. Furthermore, C2C12 myoblast cell lines were obtained from the biobank. The all cell lines were matched by age and gender (20-30 year old group, male), in order to model in vivo extracellular matrix/myocytes interaction in an in vitro system, Fibroblasts were grown to 50% confluency. In muscle biopsies interstitial fibroblasts were ranging from 20% to 70% confluency. Two frozen vials of fibroblasts from the first passage were grown in T25 flasks (Fisher Scientific, 156367) and each of them was expanded in five T25 flasks after four days. This experiment was repeated twice to obtain enough fibroblasts (50 vials) of less than five passage for the whole experiments. One frozen vial of C2C12 cells was grown in T75 flask (Fisher Scientific, 156370) and expanded into 10 flasks after three days. Each of these flasks later expanded to five flasks and frozen (50 vials) before starting differentiation by getting attach to each other from surface angels.

For fibroblast/C2C12 coculture in direct contact, Fibroblast cells were seeded in plastic slide flasks (Thermo Fisher Scientific,170920).The cells were fed with DMEM medium composed of 10% foetal bovine serum with 100 IU/ml Penicillin(Sigma-Aldrich, P4330)+100 µg/ml Streptomycin(Sigma-Aldrich, P4333). Cells were incubated in 37°C in a humidity saturated atmosphere with 5% CO2 and 95% o2.The cells were supplemented with 5 ml media every two days during growth time. 36 hours after seeding fibroblasts when the cells have around 50% confluency, they were treated to inhibit mitotic activity. The growth media was replaced with mitotic media composed of DMEM with 8µg/ml mitomycin C (Sigma-Aldrich, M0503). The cells were incubated in 37°C for two hours. Then media was removed, the cells were washed with PBS and growth media was added. 12 hours after mitomycin treatment, C2C12 cells were seeded on top of the fibroblast layer. C2C12 were fed with growth medium consisting of DMEM and 10% horse serum (Sigma-Aldrich, F0146) for 24 hours and checked for not starting attachments to each other or being confluent more than 50%. On the second day of growth the media were changed to low horse serum medium (3%) to induce differentiation in C2C12.Differentiation days counted on D0, D1…D5.The differentiation media was changed every two days during differentiation. In the day sixth of differentiation (D6),

media was removed and cells were washed with PBS. Then cells were fixed in 4% paraformaldehyde in PBS for 5 minutes at room temperature and stained with phalloidin (Thermo Fisher Scientific, 8878) to detect actin filaments. After fixation, cells were washed with PBS for three times, five minutes. One ml of 0.1% TritonX100 was added to each slide flasks for ten minutes. Cells were washed with PBS twice and one ml of 50 µg/ml 488 phalloidin conjugate solution (Sigma-Aldrich, P2141) in PBS (1%) were added to them for 40 minutes. Cells were washed three times, five minutes with PBS. Flask casquettes were removed and plastic slides were air dried. One drop of DAPI was added to each slide and coverslips were placed and pushed. After phalloidin staining, the slides were brought to the immunofluorescence microscopy unit and five images from different locations on each slide were taken.

Images were visualized with image J analyzer provided by Newcastle University. The differentiation index was determined by the number of cell nuclei in myotubes divided by the total number of nuclei in image field. Nuclei number were counted manually and repeated twice for each image.

After obtaining these results, experiments were repeated three times. Each time one flask of two mutant fibroblast cell lines, two flasks of control human dermal fibroblast cell lines from commercial and in house, One DMD adult fibroblast of in house were grown and C2C12 cells of the same passage were seeded on top of them. The whole experiment was done by one person. The examiner was blinded about source of cells in each image.

The above experiment was designed and done to show the effect of direct contact of fibroblast cell lines on C2C12 differentiation. In order to understand the paracrine effect of fibroblast/C2C12 coculture on C2C12 differentiation, the second part of this experiment was done by growing fibroblasts and C2C12 on different surfaces in one dish, sharing the same medium. Frozen vials of fibroblasts from the first passage were seeded and grown on inserted 12-well cell culture plates. Figure 2.1 shows inserted cell culture plates.

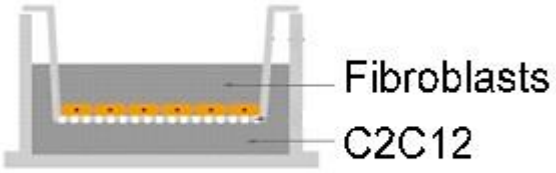


Figure 2.1.Inserted cell culture dish. Fibroblasts were grown on insert surface and C2C12 cells were grown on the bottom of the wells. Modified from (Zou *et al.*, 2008).

The fibroblasts were seeded on the top plastic insert. The cells were fed with previously described growth medium. 24 hours after seeding cells, top inserts were seen under light microscope to check cells confluency. In order to maintain same experiments conditions, mitotic treatment for fibroblasts was done when cells reach of 50% confluency according to previously described protocol. Fibroblasts were incubated in growth media for 12 hours after mitotic treatment. 200 µl of C2C12 cells were seeded on bottom layer equal to density of 50000 cell per cm2 in each well. Fibroblast growth media was replaced with high serum media (10% horse serum) in dish and inserts. C2C12 cells at the bottom layer were checked under light microscope to confirm non-attachments or overgrowth. 24 hours after seeding C2C12s, high serum medium was switched to low serum medium (3% horse serum) to induce differentiation .On the sixth day of differentiation (D6), inserts were removed and C2C12 cells were fixed with 4% PFA in PBS to start phalloidin staining. Phalloidin staining was done according to previously described protocol. Triton X100 and phalloidin antibody solutions were added at 200 µl volume on each well. Images were taken with confocal microscopy (Nikon) with a two-channel light system. Five independent images were taken from each field. Then the number of nuclei inside the myotubes was counted and the percent of whole nuclei inside the tubes in each field were divided by that to obtain C2C12 differentiation index. For two experiment differentiation index of control groups and mutant groups was compared to each other and to DMD cell line using appropriate statistical analysis.

2.16 Image J analyzer:

Image J analyzer was used to analyse images. The software was downloaded fromhttps://imagej.nih.gov.The images were taken using fixed exposure time and Z-stacks of staining were merged into a single image for capturing image. Image J quantified pixels within a defined area around a group of 4-7 cells. The cells were used in groups of 4-7 because the area around cells fora single cell was unidentifiable. For immunofluorescence experiments the pixel density around each group has been compared. For electron microscopy experiments the horizontal distance between two opposite points was measured.

2.17 Statistical analysis:

Independent t-test was used to analyse results for immunohistochemistry and electron microscopy experiments. Unpaired two proportional t-test was used for co-culture experiment analysis. All statistical analysis was done in Microsoft office excel 2016 program. P-value for statistical analysis was set at $p<0.001$***, $p<0.01$**, $p<0.05$*.

Chapter 3 collagen XII; Novel disease-causing gene in heterogenic Bethlem-like myopathy phenotype

3.1Introduction: Genetic heterogeneity in Ullrich congenital muscular dystrophy and Bethlem myopathy:

The Collagen VI-related myopathies encompass a range of neuromuscular disorders with progressive muscle weakness, skin and joint involvements. Phenotypic spectrum of these disorders as mentioned before includes severe phenotype of Ullrich congenital muscular dystrophy (UCMD) (OMIM 254090), a milder phenotype of Bethlem myopathy (BM) (OMIM 158810), intermediate phenotypes and overlapping phenotypes with Limb-girdle muscular dystrophy and myosclerosis myopathy (Allamand *et al.*, 2010).

Phenotypic diversity of collagen VI-related myopathy leads to large number of differential diagnoses. Ehlers-Danlos syndrome (OMIM 130020), Emery- Dreifus muscular dystrophy (OMIM 310300),Central core disease and fibre type I disproportion (OMIM 117000) should be considered among most important differential diagnosis for collagen VI-related myopathies (Allamand *et al.*, 2010).

The clinical manifestations of collagen VI-related myopathy is the first step to distinguish between different forms of this disorder. In the severe Ullrich form, symptoms appear during the neonatal period: hypotonia, distal joint hyperlaxity and sometimes contractures .A walking aid maybe needed for these children in the first decade of life before further progression of muscle weakness leads to wheelchair dependency. Severe Ullrich patients never learn to walk. They develop respiratory insufficiency as well (Foley *et al.*, 2013). A prominent contracture phenotype with rigid muscles, which resembles myosclerosis myopathy, has been reported in UCMD patients. In the milder form of collagen VI-related myopathy, Bethlem myopathy, a considerable diversity in the phenotype has been reported (Deconinck *et al.*, 2010). In Bethlem myopathy, contractures rarely present before the age of two years. However, hypotonia, clubfoot and torticolis may present in newborns (Baker *et al.*, 2005). Muscle weakness is less severe and could be mild. A mild presentation of proximal muscle weakness shows phenotypic overlap with different forms of Limb-girdle muscular dystrophy (Bovolenta *et al.*, 2010).

Three major genes (*COL6A1*, *COL6A2* and *COL6A3*) are responsible for most cases of collagen VI-related myopathies(Baker *et al.*, 2005).Three other *COL6A* genes (*COL6A4, COL6A5* and *COL6A6*) are expressed in the humans and other species (Sabatelli *et al.*,

2012a). These genes are homologous to *COL6A3* but their expression is more limited among non-human species. *COL6A4* expression in humanis inhibited by a chromosome break due to a large-scale pericentric inversion on chromosome three (Hicks *et al.*, 2016). In addition, in other species *COL6A4* is unable to make triple heterotrimers for secretion out of the cells (Bonnemann, 2011).So far, none of the recently discovered *COL6A (A4-6)* genes were associated with collagen VI-related myopathies. A clear phenotype-genotype correlation has been identified in *COL6A1, A2* and *A3* genes which are underlying the majority of collagen VI-related myopathies' spectrum (Zulian *et al.*, 2014).

Both severe and mild phenotypes could be inherited through dominant and recessive mutations (Lampe *et al.*, 1993; Palma *et al.*, 2009). However, severe phenotypes mainly associate with recessive mutations and milder phenotypes are mainly associated with dominant phenotypes. It should bear in mind that some dominant negative mutations could lead to severe phenotype (Lampe *et al.*, 2008).

The wide range of clinical manifestations of collagen VI-related myopathies makes the diagnostic procedure multileveled and dependent on paraclinical and genetic testing as well as history taking and clinical examinations (Foley *et al.*, 2013). So far, exome sequencing for *COL6A1, A2* and *A3* genes is the gold standard for collagen VI-related myopathy diagnosis. It is estimated that *COL6A1-3* gene mutations are responsible for around 75%-70% of patients with phenotypes resembling UCMD and 60-65% of Behtlem myopathy ("collagen VI-related myopathy" phenotype) and have been effectively ruled out in the remaining number of patients ("discovery cohort") (Bovolenta *et al.*, 2010). Collagen VI-related myopathy diagnosed individuals with no mutations in *COL6A* genes, could reflect the undiagnosis deep intronic or splice variant mutations which cannot be identified by exome sequencing (Bonnemann, 2011; Foley *et al.*, 2011). However, other affected individuals who composed the heterogenic cohort suggest that one or more additional disease-causing genes exist that can give rise to a phenotype resembling BM or UCMD if mutated (Bovolenta *et al.*, 2010).

One of the first examples of genetic heterogeneity in collagen VI-related myopathy was reported in 2004 (Ishikawa *et al.*, 2004). Ishikawa *et al* identified one UCMD phenotype from a cohort of eight affected individuals with no mutations in *COL6A1-A3* genes. All of the eight patients had signs of UCMD in their muscle evaluation. In 2005, another report of heterogenic UCMD was done through evaluation of five UCMD patients. Four of these

patients had dominant negative mutations in collagen VI (Baker *et al.*, 2005). Another report of collagen VI-related myopathy genetic heterogeneity during evaluation of MRI findings of affected individuals added to previous reports (Mercuri *et al.*, 2005). Petrini *et al* reported a UCMD phenotype in whom collagen VI mutation was excluded. However, in electron microscopy of muscle biopsies the patient demonstrated density reduction areas between reticular lamina and lamina densa with focal loss of microfibrils around basement membrane similar to collagen VI-mutant UCMDs (Petrini *et al.*, 2007). In Centre for Neuromuscular Diseases in Newcastle during developing a diagnostic immunofluorescence method for Bethlem myopathy several cases of BM with no mutations in collagen VI had been found (Hicks *et al.*, 2008). It is estimated that around 40%-50% of suspicious collagen VI-related myopathy phenotypes who were referred to the MRC Centre for Neuromuscular diseases in Newcastle are heterogenic (Debbie Hicks, personal communication). Regarding the importance of mutation detection for genetic counselling and future therapeutic approaches in these patients, finding novel disease-causing genes in heterogenic collagen VI-related myopathy cases is an emerging issue.

3.2 Aim:

In this chapter, we aim to:

Identify novel disease causing gene by exome sequencing in a cohort of patients with Bethlem-like myopathy phenotypes in whom mutations of the *COL6A1-3* genes have been ruled out previously.

Describe phenotypic spectrum and unifying clinical features of affected individuals with novel disease-causing gene.

Confirm pathogenicity of variants derived from our pipeline by bioinformatic *in silico* analysis, pedigree segregation studies and immunoblotting evaluation.

3.3 Material and methods:

3.3.1 *Clinical and genetic characteristics of a Bethlem-like myopathy cohort with no mutations in COL6A1-3 genes:*

3.3.1.1 *Patients:* A panel of thirty-six patients with Bethlem-like myopathy symptoms has been evaluated in this study. Twenty-four of these patients were referred to the MRC Centre for Neuromuscular Diseases in Newcastle since 2001. History taking, clinical

examination and genetic analysis were done in the Institute of Human Genetics (IGM) in Newcastle by medical and laboratory staffs of this centre. . Professor Kate Bushby, Professor Volker Struab and Professor Hanns Lochmuller were responsible for clinical and genetics re-evaluation of these patients in the MRC centre for Neuromuscular disorders. Twelve other patients were referred to MRC Centre for Neuromuscular Disease in Newcastle from Universitat Catholic Lueven (UCL) Centre for Neurology in Belgium (nine patients) and Oxford centre for neuromuscular disorder (Three patients). Clinical evaluation and genetic analysis had been done by medical staff of those corresponding centres. Professor Francesco Mantoni in Oxford University and Professor in UCL Belgium were responsible for first clinical and genetics evaluation of corresponding Bethelem-like patients from their centres. We received the results of these evaluations in combination with affected individuals' fibroblasts in MRC Centre for Neuromuscular Disease in Newcastle from 2013 to 2015. These patients were added to the Newcastle cohort. Professor Francesco Mantoni in Oxford University and Professor in UCL Belgium were responsible for first clinical and genetics evaluation of corresponding Bethelem-like patients from their centres.

3.3.1.2 ***Clinical and laboratory findings:*** Clinical features and individuals' information which were recorded for all affected individuals included age, gender, age at onset, hypotonia, muscle weakness, independent walking, wheelchair dependency, pattern of muscle weakness, joint rigidity and contractures, joint hyperlaxity, cardiac involvement, skin involvement, neurologic deficit, neurologic examination results, respiratory problems, scoliosis and skeletal abnormalities, CK level, Immunofluorescence staining (IF) of collagen VI in dermal fibroblasts and muscle biopsy findings.

Skin and muscle biopsies for all of these affected individuals from Newcastle cohort were collected in Newcastle medical school affiliated hospitals. Samples were kept in biobank of MRC Centre for neuromuscular Diseases in Newcastle and dermal fibroblasts were extracted from those samples. Collagen VI IF staining in dermal fibroblasts was done in research laboratories affiliated to the MRC Centre for Neuromuscular Diseases in Newcastle. Muscle staining was done in Muscle Immunology Unit (MIU) of MRC Centre for Neuromuscular Diseases in Newcastle. Figures 3.1, 3.2 and table 3.1 represented clinical and paraclinical characteristics of these patients.

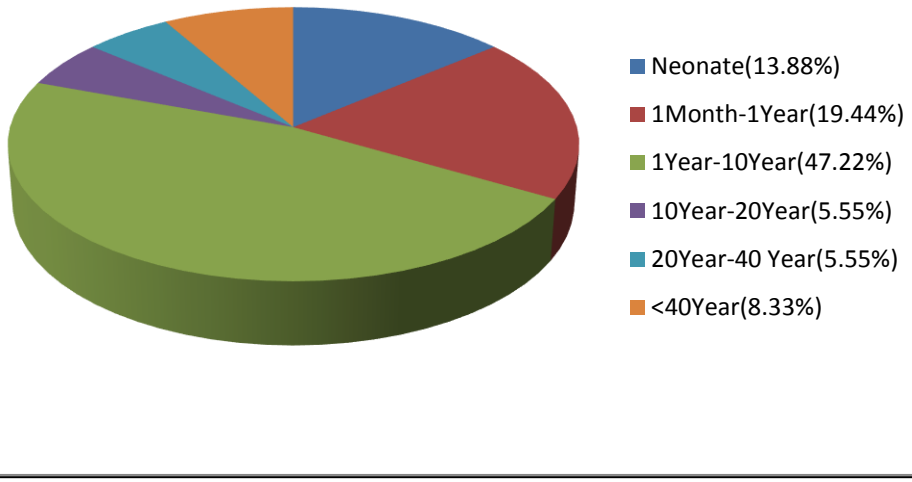


Figure 3.1: Age of onset in affected individuals. The largest number of patients is represented in the third age group which is 1-10 year-old individuals.

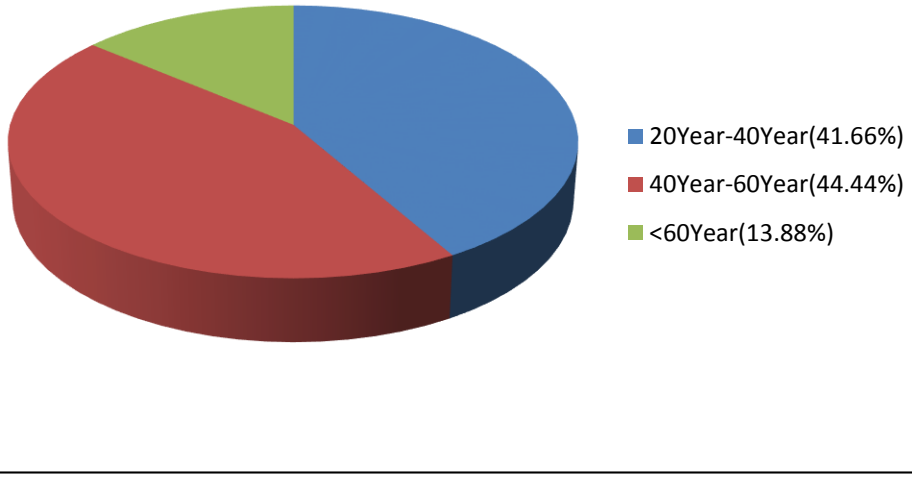


Figure 3.2: Affected individuals' current age. Current age is shown in different age groups. The largest number of patients is in the "between-40-to-60-years" group.

Column1	Sex	Ambulation status	Pattern of muscle weakness	Disease progression	Contractures	Join hyperlaxity	Skeletal abnormalities
P1	F	Poor at sports at childhood	Mild proximal weakness	Mild	LFF	Upper limb joint hyperlaxity, hip dislocation	Normal
P2	F	Ambulant	Minimal weakness	Mild	Torticolis, newborn LFF, elbow, knees	Generalized hyperlaxity Hip subluxation	Normal
P3	M	Walking aid	Proximal weakness	Mild to moderate	LFF, TA, wrist flexion, knee	NR	Normal
P4	M	Ambulant	Proximal weakness	Mild	No	Normal	Rigid spine
P5	M	Ambulant	proximal weakness, trendelenburg gait	Mild	LFF, TA, elbow	Normal	Rigid spine
P6	M	Ambulant	Generalized weakness	Stable	LFF, TA, elbow, Neck	NR	Kyphoscoliosis
P7	M	Ambulant	Proximal weakness	Mild	LFF, TA, elbow, hip	NR	Normal
P8	M	Ambulant	Proximal weakness	Moderate	LFF, TA, elbow	Hypotonic infant	Normal
P9	F	Walking with a frame walk	Mild facial and proximal weakness	Mild	LFF, elbow	Hypotonic infant	Kyphosis
P10	F	Wheelchair dependent	Mild weakness	Mild	LFF	Elbows and knees	Normal
P11	M	Walking short distance	Generalized weakness	Stable	LFF, TA. elbow	NR	Normal
P12	F	Struggling getting up from floors	Generalized wasting of muscle	Mild	LFF, TA, hips, elbow	NR	Normal
P13	F	Ambulant	Symmetrical limb weakness	Stable	LFF elbow, hip, Neck	Recurrent jaw laxation	Rigid spine
P14	F	Difficulty walking	Upper limb weakness	Mild	LFF, TA, elbow	Recurrent patellar dislocation	Normal
P15	M	Wheelchair dependent	Neck weakness	Severe	LFF, TA, elbow	NR	Normal
P16	F	Difficulty running and climbing	Generalized weakness	Mild	LFF, TA, elbow, wrist extension	NR	Normal

P17	F	Ambulant	Neck flexion weakness	Mild	LFF, TA, elbow	NR	Kyphosis
P18	M	Difficulty getting up from floors	Proximal Weakness	Mild	LFF, wrist, rigid lumbar spine	NR	Normal
P19	F	Ambulant	Proximal weakness	Stable	LFF, TA, elbow	NR	Normal
P20	M	Difficulty getting up from floors	Generalized weakness	Mild	LFF, TA, hips	NR	Normal
P21	M	Difficulty walking	Generalized weakness	Mild to moderate	LFF, TA, shoulder	NR	Rigid spine
P22	F	Frequent fall	Proximal weakness	Stable	LFF, TA	NR	kyphoscoliosis
P23	F	Unable to keep up with peers	Proximal lower limb weakness	Mild	LFF, TA elbows	NR	Scoliosis
P24	F	Ambulant	Mild weakness	Stable	Torticolis, LFF, elbow, knees	Jaw subluxation	NR
P25(ox)	M	Difficulty getting up from floors	Proximal limb weakness	Mild	LFF, elbow	MCP hyperlaxity	NR
P26(ox)	M	Difficulty getting up from floors	Proximal limb weakness	Mild to moderate	LFF	MCP hyperlaxity	Normal
P27(ox)	F	Ambulant	Mild generalized weakness	Stable	NO	Normal	Normal
P28(UCL)	F	Walking short distance	Mild to moderate weakness	Moderate	LFF, TA, wrist	Normal	Normal
P29(UCL)	M	Ambulant	Proximal weakness	Stable	NO	Normal	Normal
P30(UCL)	F	Ambulant	Facial weakness	Stable	LFF, TA, hip	NR	Normal
P31(UCL)	F	Ambulant	Facial weakness	Stable	LFF,KNEE	NR	NR
P32(UCL)	F	Difficulty getting up from floors	upper limb flexion weakness	Mild to moderate	LFF,TA	MCP hyperlaxity	Rigid spine
P33(UCL)	M	Difficulty getting up from floors	Wasting of upper arms and shoulders	Mild to moderate	LFF, elbow	MCP hyperlaxity	Scoliosis
P34(UCL)	M	Walking short distance	Generalized weakness	Mild	TA, elbow, hip	NR	Normal

P35(UCL)	M	Difficulty running and climbing	Generalized weakness	Moderate	TA, elbow	Patellar dislocation	Normal
P36(UCL)	M	Ambulant	Proximal lower limb weakness	Stable	TA	Patellar dislocation	Normal

Column 1	Skin abnormalities	Respiratory involvement	Cardiac involvement	Collagen VI IF	CK	Muscle biopsy	Family history	Genetic analysis
P1	Normal	Normal	Normal	Normal	93	NR	Positive	LMNA,CP
P2	Hyperkeratosis pilaris	Normal	Normal	Normal	747	Myopathic, fibrosis	Positive	CP
P3	Hypertrophic scar	Normal	Normal	Normal	680-973	Myopathic, fibrosis	Positive	LMNA,DM2, PROMM,FSHD,CP
P4	Hypertrophic and atrophic scar	Normal	Normal	Normal	1310	NR	Positive	None
P5	Hypertrophic and atrophic scar	Normal	Normal	Normal	824	NR	Positive	None
P6	Possible atrophic scar	Reduced vital capacity	Partial RBBB	Normal	500	Myopa thic, prominent normal fatty replacement	Negative	FHLI, LMNA, CP
P7	Normal	Reduced vital capacity	RBBB, pacemaker	Normal	230	Myopathic, prominent fatty replacement	Negative	LMNA,CP
P8	Psoriasis	NH	Normal	Normal	992	Myopathic, 50%fat replacement	Negative	LMNA, FKRP,CP
P9	NR	NH	Normal	Normal	Normal	Mixed myopathic, Neurogenic	Negative	LMNA, FSHD,CP
P10	Normal	Normal	Normal	Normal	67	Myopathic	Negative	SMN,CP
P11	Generalized lipodystrophy	NH	Normal	Normal	1301	Myopahtic, type I fibre grouping	Negative	LMNA,CP
P12	Hyperkeratosis pilaris	NH	Normal	Normal	395	Myopathic, myosclerosis	Negative	FHLI, CP
P13	Slow healing wound	NH	NR	Normal	1891	Myopahtic	Positive	CP

P14	Prominent scar	Normal	Normal	Normal	589	NR	Negative	LMNA
P15	Prominent scar	Normal	Normal	Abnormal	800-2645	NR	Negative	LMNA,CP
P16	Hypertrophic scar	NH	AV block	Abnormal	823	Myopathic, type 2 abnormal predominance	Negative	LMNA,CP
P17	Hypertrophic scar	Reduced vital capacity	Normal	Abnormal	253-500	Myopathic, laminin B1 abnormal reduced	Negative	CP
P18	Hyperkeratosis pilaris and easy bruising	Normal	Normal	Abnormal	1110	NR	CAPN3	FKRP,CAPN3,CP
P19	Prominent scar, hyperkeratosis pilaris	Normal	Normal	Abnormal	588	Myopathic, fibrosis	Negative	LMNA,CP
P20	Atrophic scar	Normal	Normal	Abnormal	214	Fibrosis	Negative	LMNA,CP
P21	Hyperkeratosis pilaris	NR	NR	Abnormal	52	NR	Negative	LMNA,CP
P22	Hyperkeratosis pilaris	Normal	Normal	Abnormal	Elevated mixed	NR	Negative	LMNA, FKRP,CP
P23	Normal	Normal	Normal	Abnormal	654	Myopathic	Negative	LMNA, SEPN1,CP
P24	Normal	NR	NR	Abnormal	340	Myopathic	Negative	LMNA,CP
P25(ox)	Atrophic scar	NR	NR	Abnormal	NR	Myopathic	Positive	FKRP,CP
P26(ox)	prominent scar	NR	NR	Abnormal	NR	Myopathic	Positive	FKRP,CP
P27(ox)	Keloid	NR	NR	Abnormal	NR	Fibrosis	Positive	LMNA,CP
P28(UCL)	Hypertrophic scar	NR	Normal	Abnormal	NR	NR	Negative	LMNA, FKRP,CP
P29(UCL)	Hypertrophic scar	NR	Normal	Normal	NR	NR	Negative	NR
P30(UCL)	Normal	NR	Normal	Normal	NR	NR	Negative	NR
P31(UCL)	Normal	NR	Normal	Normal	NR	NR	Negative	NR
P32(UCL)	Hyperkeratosis pilaris	NR	Normal	Abnormal	NR	Myopathic	Negative	NR
P33(UCL)	Hypertrophic scar	NR	Normal	Abnormal	NR	Myopathic	Positive	NR

P34(UCL)	Normal	NR	Normal	Abnormal	NR	NR	Positive	LMNA,CP
P35(UCL)	Normal	NR	Normal	Abnormal	NR	NR	Positive	FKRP,CP
P36(UCL)	Normal	NR	Normal	Normal	NR	NR	Negative	LMNA,CP

Table 3.1 Clinical features of Bethlem-like myopathy cohort with no mutations in *COL6A1-A3.*

As the figures demonstrate the largest number of patients had been diagnosed in their first decade of life after the neonatal period. According to the table the majority of patients survived till late adulthood and remain ambulant which represents a mild disorder and a slow progression of the disease.

The most common symptoms of collagen VI-related myopathy had been checked in these patients. Joint contractures, abnormalities in muscle biopsy and a high creatine kinase level are the most frequent symptoms among the patients. Symptoms such as spine abnormality and skin involvement which are more specific for collagen VI-related myopathy present in less than half of the patients. Respiratory symptoms such as reduced Forced Vital Capacity (FVC) and hypoventilation have been seen in about thirty five percent of patients, while cardiac symptoms are very rare and only present in one or two patients. Unreported or rare symptoms of neuromuscular disorders such as lipodystrophy and seizures are reported. Systemic diseases like hypertension and diabetes had been reported in two affected individuals.

3.3.1.3 *Genetic analysis:*

Collagen VI-related myopathy in particular Bethlem myopathy as a differential diagnosis was suggested for these patients and a genetic examination was done. The exome sequencing for *COL6A1,A2* and *A3* was done once in Northern Genetics Service in Newcastle by NHS genetic laboratory staffs for diagnostic purposes and again in Centre for Genetic Analysis in Oxford University by genetic laboratory staffs register these patients in a research data gathering all Bethlem -like patients with no *COL6A1-A3* mutations.*COL6A1-A3* variants which derived from Sanger sequencing in Northern Genetics Service in Newcastle was found to be non-pathogenic by laboratory staffs according to *In silico* analysis. *In silico* analysis was done using mutation taster (http://www.neurocore.charite.de/MutationTaste) and polyphen (http://www.genetics.bwh.harvard.edu/pph2) for variant analysis and predicted pathogenicity. In addition, 1000 genomes databases (www.internationalgenome.org) and the NHLBI Exome Sequencing project (EVS) (evs.gs.washington.edu) were used to exclude polymorphisms from rare pathogenic variants.

Genetic analysis for Limb-Girdle Muscular Dystrophy, Ehlers-Danlos syndrome and Myasthenia Gravis was done in part of the cohort by genetic laboratory staff in Northern Genetic Service in Newcastle (table 3.1).Based on literature reviews several newly discovered genes for neuromuscular disorders were also investigated. Ten candidate genes for heterogeneity in collagen VI, namely *PLOD3*, *P4HA1*, *P4HA2*, *P4HA3, BGN*, *DCN*, *PLOD1*, *PLOD2*,*P4HB* and *COL5A1*, were Sanger sequenced in Newcastle cohort patients' by Dr Debbie Hicks prior to this study(Hicks *et al.*, 2014). All the variations which were found in these genes were deemed non-pathogenic by *in silico* analysis. Family pedigree evaluation suggested autosomal dominant mode of inheritance for most of the affected individuals. After discovering the novel *COL12A1* mutations in two patients of these five pedigrees, by Golara Torabi Farsani another *COL12A1* mutation was discovered in a patient from undiagnosed cohort of 20 individuals with neuromuscular disorders. These patients also had been referred to Newcastle Centre for Neuromuscular disorders in Newcastle from 2001 and had been evaluated by our medical staff and Doctors. The whole exome-sequencing (WES) was done in these patients on 2012 at Decode institute. The bioinformatics analysis of WES was done in MRC centre for neuromuscular disorder in Newcastle by Dr Debbie Hicks and Dr Steven Laval. The detailed information of patient analysis for this cohort was given in Material and Method chapter of this research (section 2.6).

The novel *COL12A1* patient who we call it BM1` will be described later. The reason he was not included in the first Bethlem-like myopathy patients with no mutations in *COL6A1-A3* genes was that clinical presentation of this patient is different from other collagen VI-related patients. The symptoms such as lipodystrophy in this patient was not reported in other Bethlem-like myopathy patients before.

3.3.2 *Clinical and genetic analysis of five overlapping BM phenotype myopathy cohort with no mutations in COL6A1-A3:*

3.3.2.1 *Clinical features and corresponding pedigree:*

Five patients from the previously described Bethlem-like phenotype cohort were selected. The selection criteria for these patients included the most overlapping

phenotype with BM, homogeneity with other selected members in disease symptoms and progression, availability of patients and their biologic samples, clear pedigree and family history, availability of parental DNA, negative Sanger sequencing and linkage analysis for *COL6A1-A3* genes, with normal immunofluorescence staining for collagen VI. This selection was done by principal investigators of MRC centre for Neuromuscular disoders prior to this research. Figure 3.3 shows the pedigree of each individual patient.

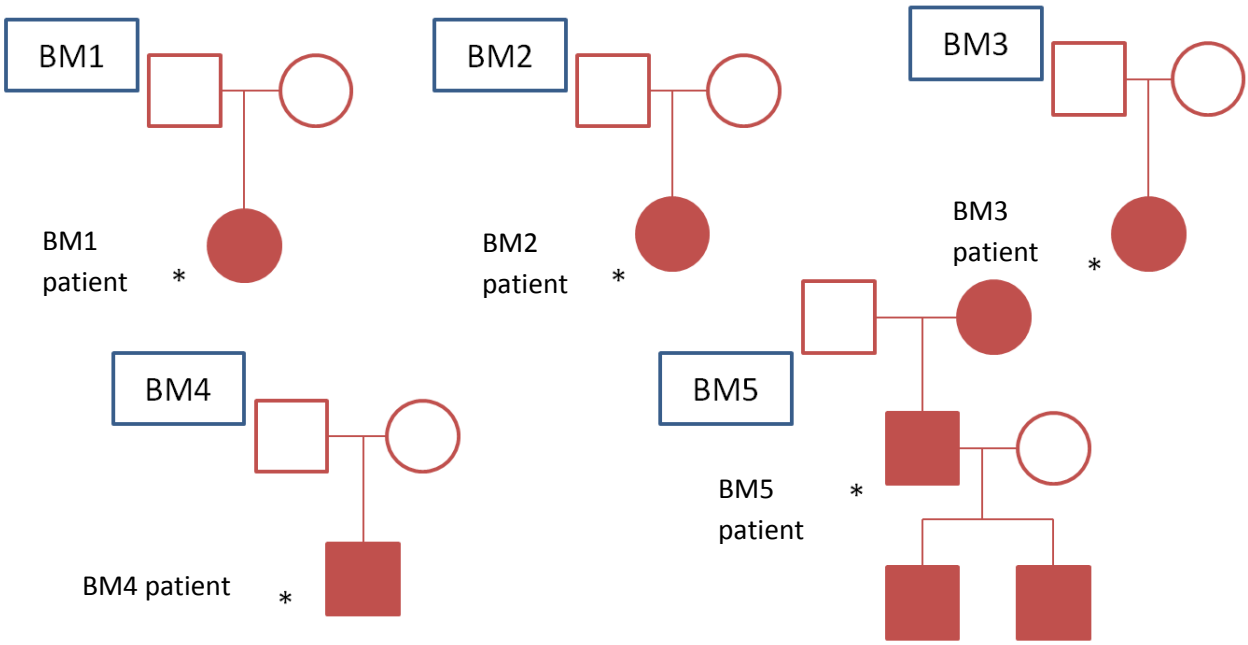


Figure 3.3: Five pedigrees of Bethlem-like patients. Three pedigrees demonstrates autosomal dominant de novo pattern of inheritance while BM2 and BM5 are autosomal dominant inherited. The star (*) shows proband which genetically more evaluated in our research.

Table 3.2shows the clinical features and immunofluorescence staining results for dermal fibroblasts for each Bethlem-like patient.

Patients (The pedigree related number)	First presentation	Main clinical findings	Fibroblasts IF results
Bethlem-like 1(BM1)	Infancy. Delayed motor milestones, walking difficulties.	Finger flexion and elbow contracture, Proximal muscle weakness	Normal
Bethlem-like 2 (BM2)	Infancy. Delayed motor milestones.	Hyperlaxity, Muscle weakness	Normal
Bethlem-like 3 (BM3)	Infancy. Difficulty in walking.	Joint hyperlaxity, Proximal muscle weakness	Normal
Bethlem-like 4 (BM4)	Childhood. Delayed motor milestones.	Finger flexion and elbow contracture. Muscle weakness	Normal
Bethlem-like 5 (BM5)	Childhood. Difficulty in walking and stair climbing.	Finger flexion and elbow contracture, Proximal muscle weakness	Normal

Table 3.2 Clinical features of five non-collagen VI-related Bethlem-like patients.

3.3.2.2 *Genetic analysis:*

Genomic DNA of these patients was extracted from peripheral blood and sent for whole exome sequencing (WES), which was done according to the Illumina protocol that was described in detail in the material and methods chapter before. The first step for the selection of variants, all non-synonymous, non-sense and splice site and very rare variants in other collagen genes included *COL22, COL2, COL6A6, COL15A1, COL12A1, COL4A3* were chosen. For each pedigree more than two hundreds rare, non-synonymous variants were derived from the pipeline. Computer based analysis revealed no common variant in at least three pedigrees. The variant calling section was done by DR steven Laval. We decided to focus on all collagen variants in each pedigree in the first step. The existence of the variants and their

pattern of inheritance in corresponding pedigrees were confirmed by Sanger sequencing by Golara Torabi Farsani. Figure 3.4 shows the Variant calling for each pedigree.

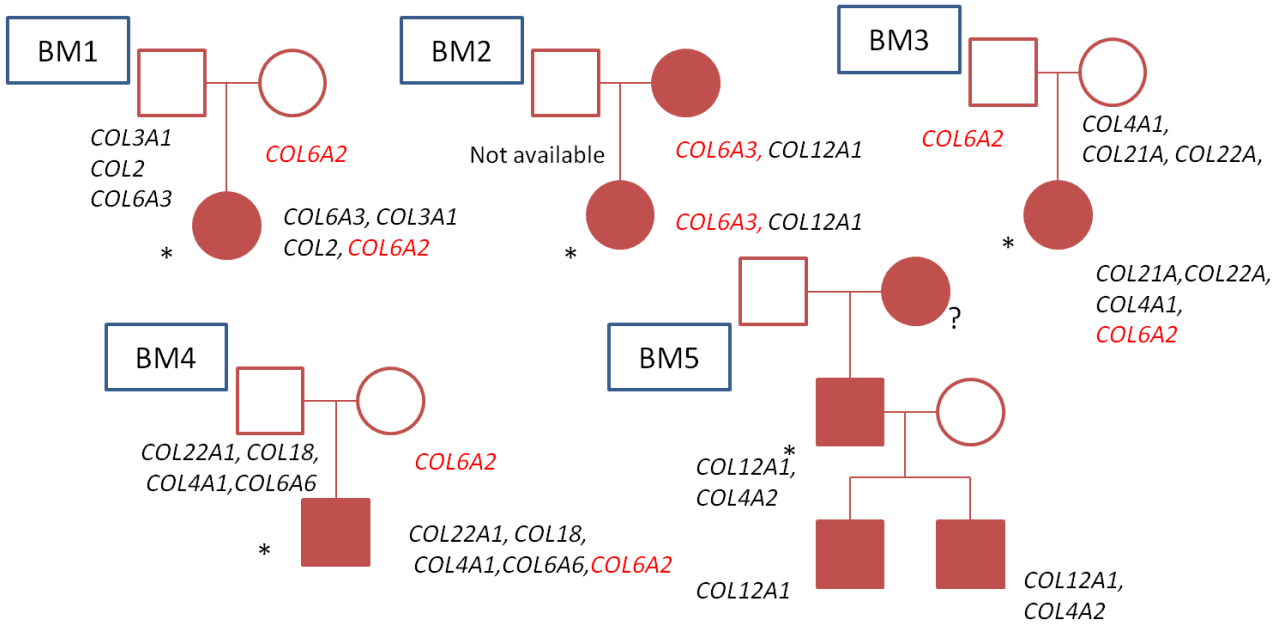


Figure 3.4.Variant calling in each pedigree. Segregation of selected variant after variant calling and *in silico* analysis in corresponding pedigrees. Variants in red has the frequency more than 0.005 according to 1000 genome study and all are polymorphism.

None of the variants appeared as *de novo* mutations in the pedigrees for BM1, BM3 and BM4. In those corresponding pedigrees mutations present in affected individuals were inherited from a non-affected parent. However, in BM2 and BM5, two very rare variants in the gene *COL12A1* were discovered which segregated with the disease in corresponding pedigrees. These two variants and their features will be described later. Appendix A shows the list of primers and variants which were sequenced.

3.4 Result:

3.4.1 *Discovery of threeCOL12A1 variants in corresponding pedigrees of three Bethlem-like affected individuals:*

Two affected individuals from previously described pedigrees (BM2 and BM5) and one affected individual from undiagnosed neuromuscular cohort (BM1') identified with *COL12A1* mutations derived from exome sequencing and verified by Sanger sequencing. These variants completely inherited with autosomal dominant pattern of inheritance. The details of these mutations will be described below. Appendix B shows primers for these mutations.

3.4.1.1 *Genetic analysis in BM2:*

The first new mutation (NM: 004370 c.8357A>G: p.Gly2786Asp) was identified in pedigree of BM2. Figure 3.5 shows pedigree and sanger sequencing result for this patient.

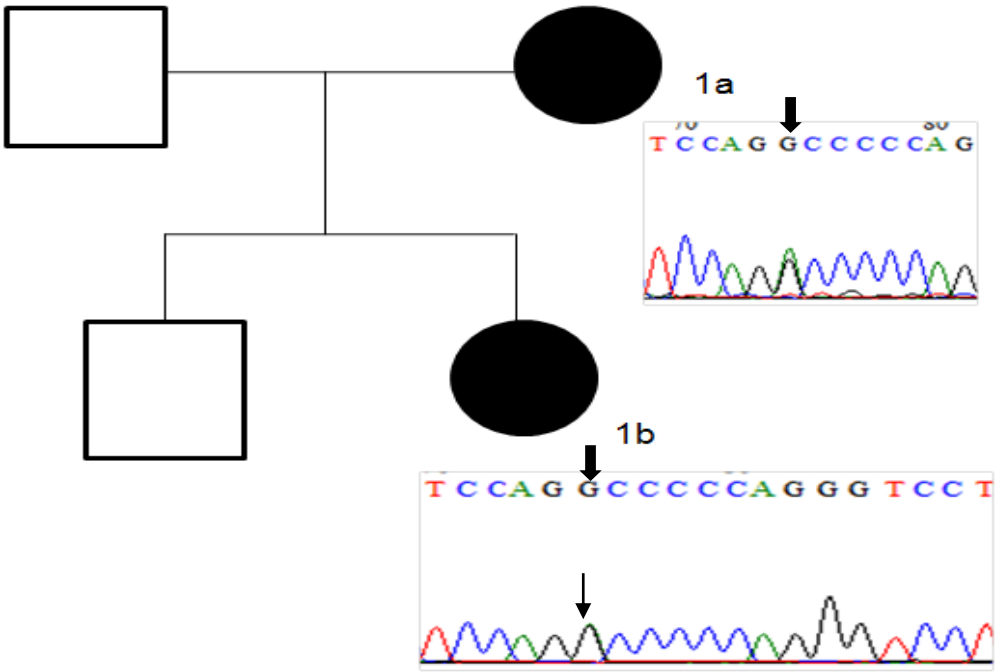


Figure 3.5: mutation in BM2. Segregation of p.Gly2786Asp (BM5) with autosomal dominant pattern in corresponding pedigree. The black wide arrows represent the site of substitution. The black narrow arrow point at green shade of Adenine signal parallel with Guanine signals. DNA for unaffected members was not available.

This change causes a substitution of the neutral amino acid residue (Glycine) with a negatively charged residue (Asparatic acid) in a fibronectin type III domain of the

protein. This substitution occurred in a conserved Gly-X-Y motif which is known to be very important in the post-translational assembly of collagen trimers .A failure in the trimer assembly prevents the secretion of collagen chains to the extracellular matrix. These changes are reported to be pathogenic in other collagens including*COL6A*genes. This variant is not reported in the 1000 genomes database (www.internationalgenome.org), NHBLI exomes sequencing project (EVS) (https://esp.gs.washington.edu/drupal) or the ExAc database (http://exac.broadinstitute.org). *In silico* analysis with Mutation Taster (http://mutationtaster.org) predicted that this change could be pathogenic and showed that the residue at this position is highly conserved among mammalians. These *in silico* analysis and genetic sequencing was done by Golara Torabi.

3.4.1.2 *Genetic analysis in BM5:*

The second variant in the *COL12A1* gene (NM: 004370 c.5893T>C: p.Arg1965Cys) was found in a family with three affected members (BM5). This variant segregated with the autosomal dominant pattern of inheritance in the corresponding pedigree. Figure 3.6 shows pedigree and sanger sequencing result for these patients.

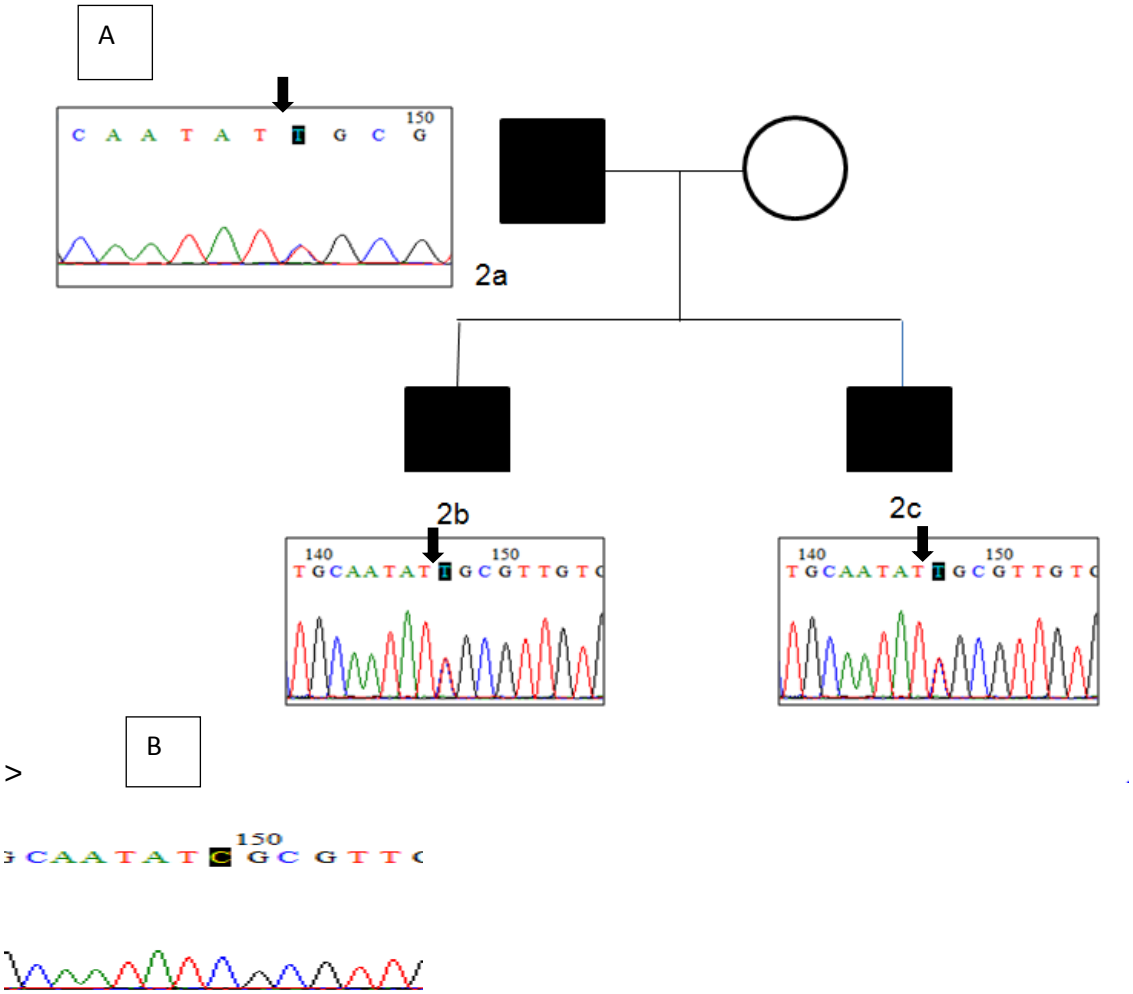


Figure 3.6: *COL12A1* mutation in BM5. A) Segregation of c.5893C>T: p.Arg1965Cys (BM5) with an autosomal dominant pattern in the corresponding pedigree. The substitution nucleotide site is highlighted in black. B) Control sequence for the healthy member of family.

In silico analysis revealed that the p.Arg1965Cys variant is located in a fibronectin III domain of collagen XII which is predicted to be important in the attachment of proteins to other extracellular matrix components. The addition of a cysteine in the polypeptide chain of collagen XII would also lead to an unpaired cysteine which could form aberrant disulphide bonds.

Further investigation through Mutation Taster tool predicted the change to be a disease-causing variant. This change is detrimental for the whole collagen structure because it could produce a new disulphide bond. This change is present at a low frequency (0.016%)I n the NHBLI Exome Sequencing project(EVS)but absent in the 1000 genomes databases. This frequency is only reported for Afro-american population.

The ExAc database shows a frequency of 0.000083641 for this variant. These *in silico* analysis and genetic sequencing was done by Golara Torabi.

3.4.1.3 *Genetic analysis in BM1':*

In addition to the previous Bethlem-like myopathy phenotype, exome analysis in a cohort of undiagnosed muscle disorder patients revealed a heterozygous 5'splice donor site mutation (*COL12A1:g.C75815009T*) at the start of exon 53 of *COL12A1* in one patient. In this research we called this patient BM1'. RT-PCR and *in silico* analysis confirmed a heterozygous in-frame deletion of *COL12A1* exon 54 (c.del:8345-8431het) which translated to a conserved region in the collagenous domain of collagen XII. Collagenous domains are important in collagenous features of the protein and are unique for each collagen molecule (Halper and Kjaer, 2014). This change does not exist in the NHBLI exome sequencing project (EVS), 1000 genomes database and ExAC database. Segregation analysis revealed that it is an autosomal dominant *de novo* mutation. This mutation does not appear in other members of the family. According to non-overlapping phenotype of this patient which will be described later which included lipodystrophy, mutations in group of genes which mostly caused lipodystrophy were evaluated. Exome sequencing results for mutations in *LMNA, ZMP, STE24, PLIN, PPARG, CIDEC, AGPAT2, BSCL2, CAV1, PTRF* genes were investigated which did not reveal any new pathogenic mutations. The investigation was done by Dr Ana Topf according to exome sequencing results In addition, the whole exome sequencing result was investigated in this patient according to pipeline which was described before. No other pathogenic mutation was derived from this investigation. Figure 3.7 shows the genetic analysis result in this patient.

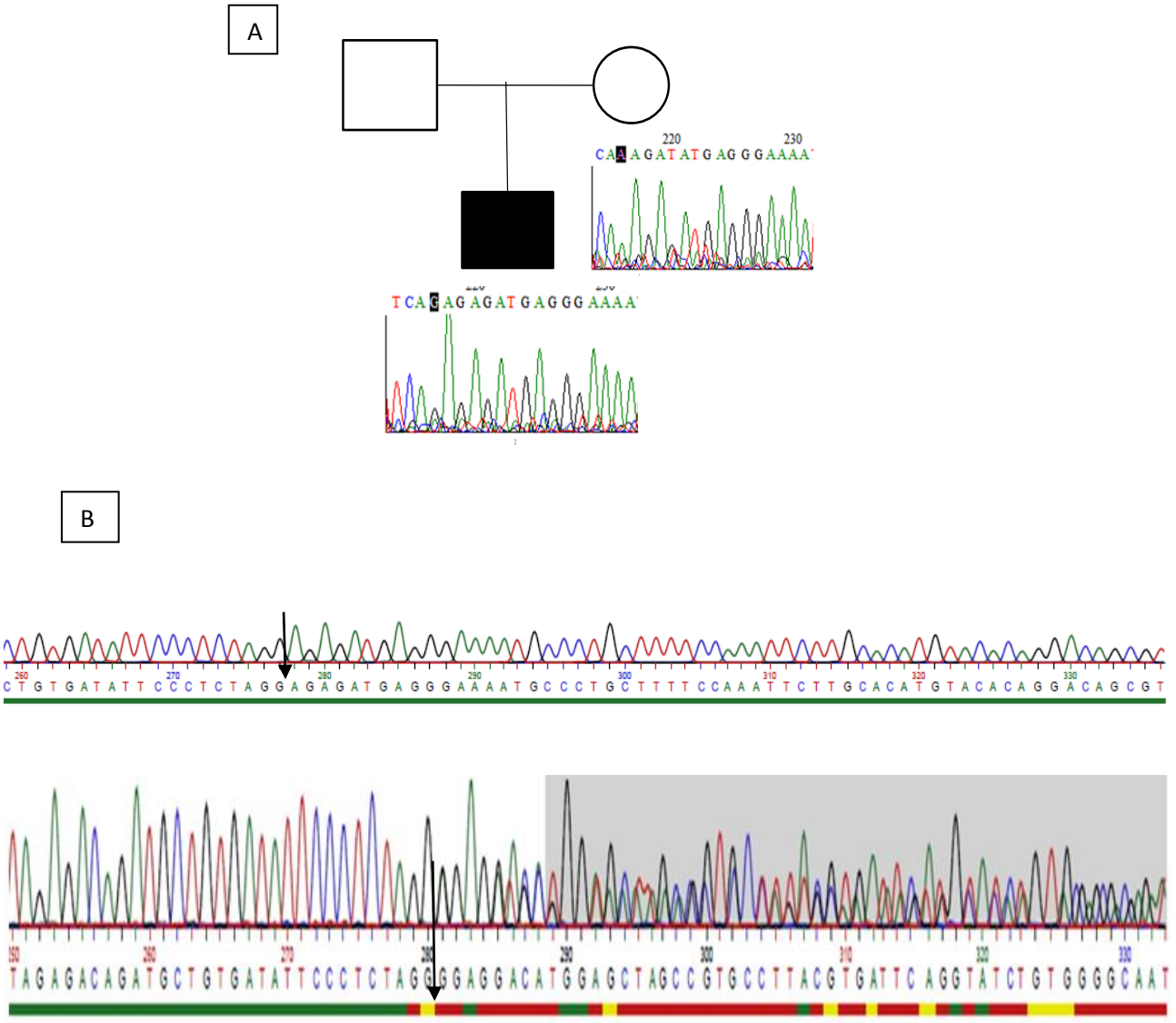


Figure 3.7. Genomic analysis in BM1'.A) Genomic DNA analysis is shown the result for g. 75815009C>T (BM1'). B) cDNA sequencing for this patient is shown del: c.8345-8432del: p.2727-2755del (BM1') in normal individual and mutant patients. The black arrows represent the start of missplicing section.

3.4.1.4 *Collagen XII protein schematic picture:*

The novel described mutations in *COL12A1* are localizing in different domains of collagen XII protein. The schematic picture 3.8 demonstrates location of each of these mutations.

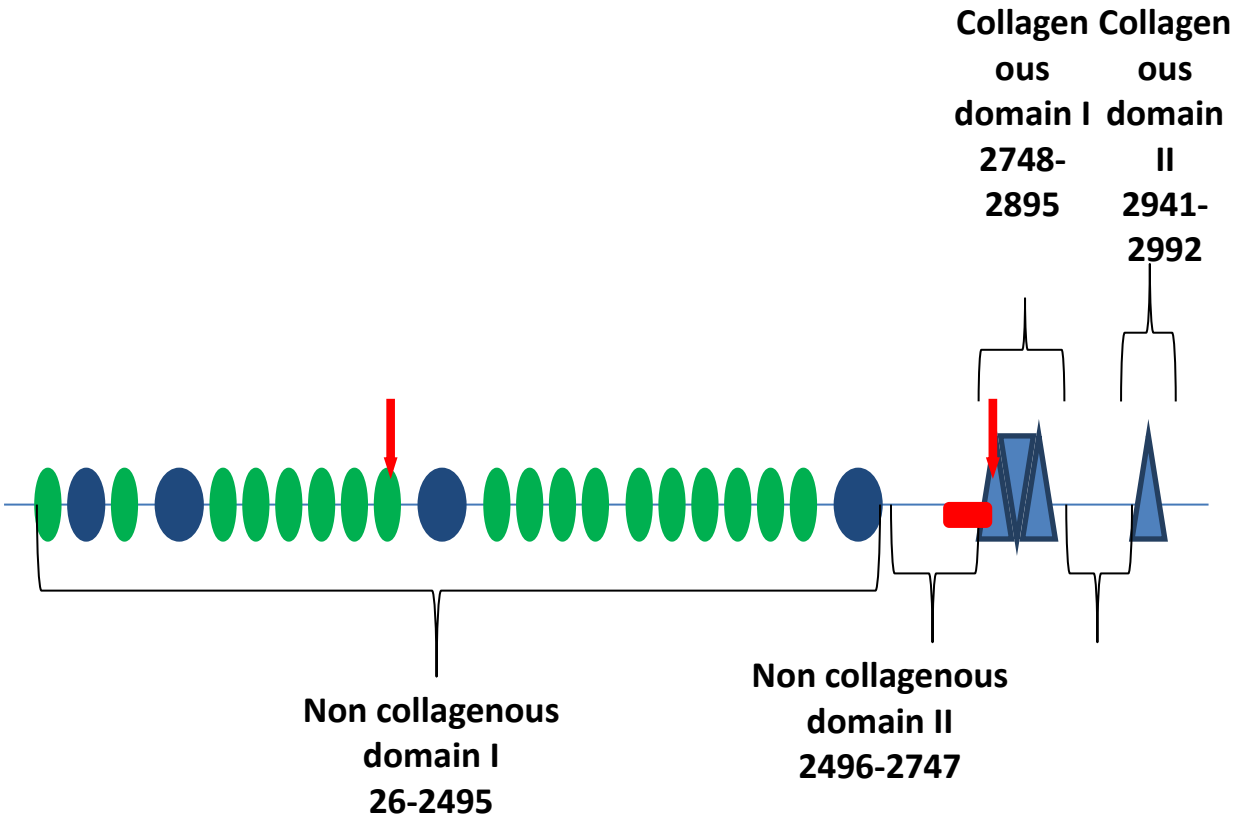


Figure 3.8.Schematic picture of collagen XII. Collagen XII molecule consists of two non-collagenous and two collagenous domains. The non-collagenous domains consists of eighteen fibronectin type III domains and four vonwilbrand A domains. The first red arrow shows the location of missense mutation in BM5 which is in Fibronectin type III domains. The red horizontal line shows location of splice variant in BM1' which leads to remove part of collagenous first domain. The second arrow demonstrates the location of missense mutation in BM2 which is located in collagenous part of the protein.

3.4.1.5 *COL12A1 exome screening in the cohort of Bethlem-like myopathy patients with no mutations in COL6A1-A3 genes:*

After finding three novel mutations in the gene *COL12A1* from the result of WES, this gene was sequenced in the cDNA level in the rest of the Bethlem-like myopathy patients with no mutations in *COL6A1-3* genes cohort. After excluding polymorphisms and false positive variants no additional mutations in *COL12A1* were found in the rest of the cohort. A list of *COL12A1* primers and novel variations is attached in the appendix C.

3.4.2 *Detailed clinical review of COL12A1 mutant individuals:*

3.4.2.1 *Clinical review of BM2 and BM5 affected family:*

Generalized muscle weakness in childhood was the unifying aspect of all five patients. In the BM2 family, the affected child had torticolis and hypotonia in her neonatal period. Later she presented kyphosis and distal hyperlaxity, delayed motor milestones and skin involvement in form of hyperkeratosis pilaris. Finger flexor contracture as common sign of Bethlem myopathy in conjunction with knee and elbow contracture are presented in child. Her mother also presented finger flexion, joint contractures and muscle weakness. She reported less ability for sport in childhood. Collagen VI immunofluorescence staining was normal in both affected individuals. Mild to moderate CK level and myopathic features as well as fibrosis and type I fibre grouping were found in mother. In the BM5 family, the father suffered from muscle weakness in infancy which presented in difficulties in sport during school time, low scores on physical activity and being unable to finish races. He reported a relief in muscle power during the teenage period which is consistent with reports from four other affected individuals. The muscle weakness deteriorated during his 30s. Gradually he lost the ability to climb stairs, walk independently or stand up from a low chair. His two sons (ages23 and 26 years old) complained about muscle weakness. They had finger contractures and skin scarring. Skeletal abnormalities such as a winged scapula and mild scoliosis were found in one patient (BM5 family, father). Figure 3.9 shows some of the clinical presentations in these patients.

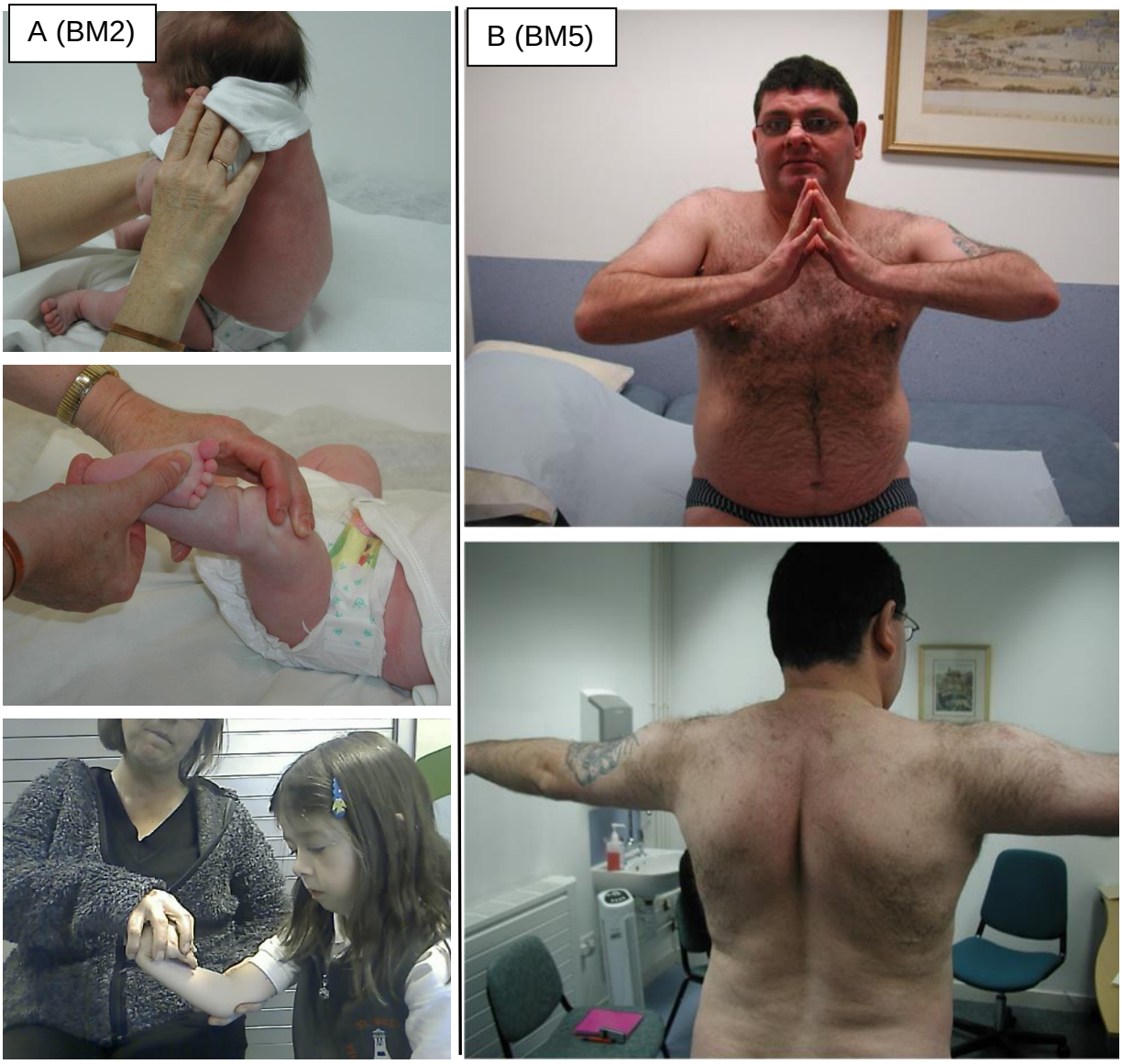


Figure 3.9. Clinical features in BM2 and BM5.A) Clinical features of affected patient in pedigree BM2 which is included joint hyperlaxity and spine deformity and B) Clinical features of affected patients in pedigree BM5 which is included Interphallangeal joint deformity and skin scars (Hicks *et al.*, 2014).

3.4.2.2 *Clinical review of BM1':*

The patient is a 21-year-old British male with neonatal hypotonia and bendy joints. The parents reported delayed gross motor development during infancy, which improved with age. Joint hyperlaxity was one of the first symptoms in the patient which led to recurrent knee dislocation and a consequent operation. The patient's

first medical evaluation was in 2008 because of back pain and patellar dislocation. Skin involvement such as a hypopigmented patch, mild contractures on finger flexors and scoliosis was found during the last evaluation in the MRC Centre for Neuromuscular Diseases in Newcastle. Generalized lipodystrophy and quadriceps muscle hypertrophy were unique features which are not seen in other patients. A mild muscle weakness was seen during physical evaluation but the patient does not complain about low muscle force. The muscle biopsy from the quadriceps was reported normal. Before including the patient in an undiagnosed cohort of neuromuscular disorder in Newcastle, genes such as *PHL1*, *Lamin A/C, PTR-CAVIN*, and Pompe disease were sequenced in the patient. Laboratory evaluation also showed a high CK level (1301) in the patient. Figure 3.10 shows the clinical features of the patient.

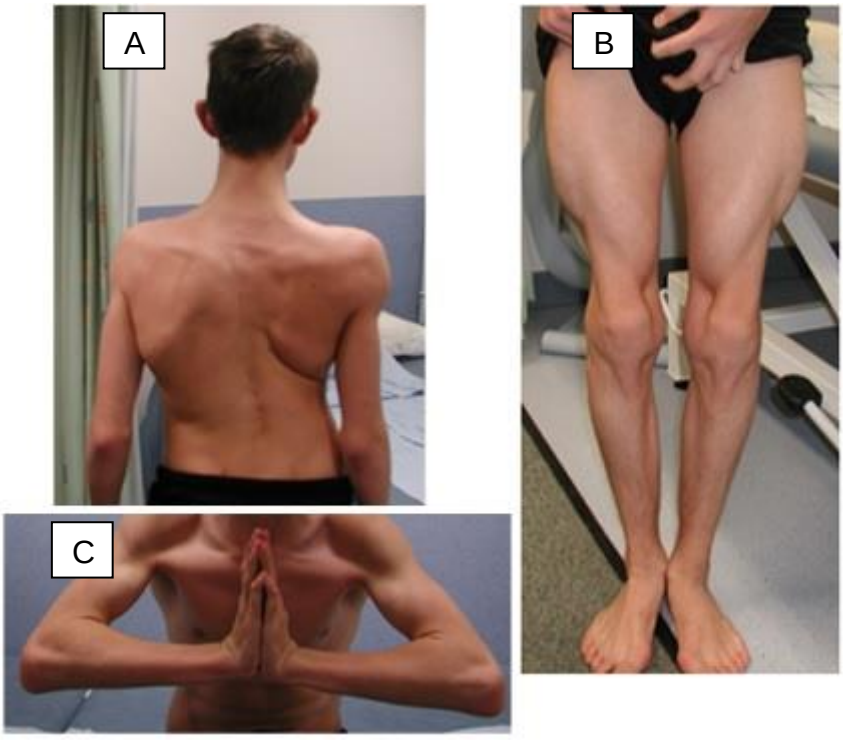


Figure 3.10. Clinical features of BM1'. New patient with g. 75815009C>Tdel: : c.8345-8432del, p.2727-2755del (BM1') mutation. Scoliosis (A), Lipodystrophy with less distribution of fat in calf and knee areas (B) and finger flexion contractures (C) is presented in this patient.

3.4.3 *Immunofluorescence staining in COL12A1 mutant individuals:*

3.4.3.1 *Immunofluorescence staining for collagen XII in dermal fibroblasts of COL12A1 mutant individuals:*

Immunofluorescence staining was done by optimization of original protocol reported by Hicks et al (Hicks *et al.*, 2008). The staining was repeated three times and five images were captured from each slides. The images were captured by Zeiss immunofluorescence microscope. Figure 3.11 demonstrates the result of collagen XII IF staining in dermal fibroblasts.

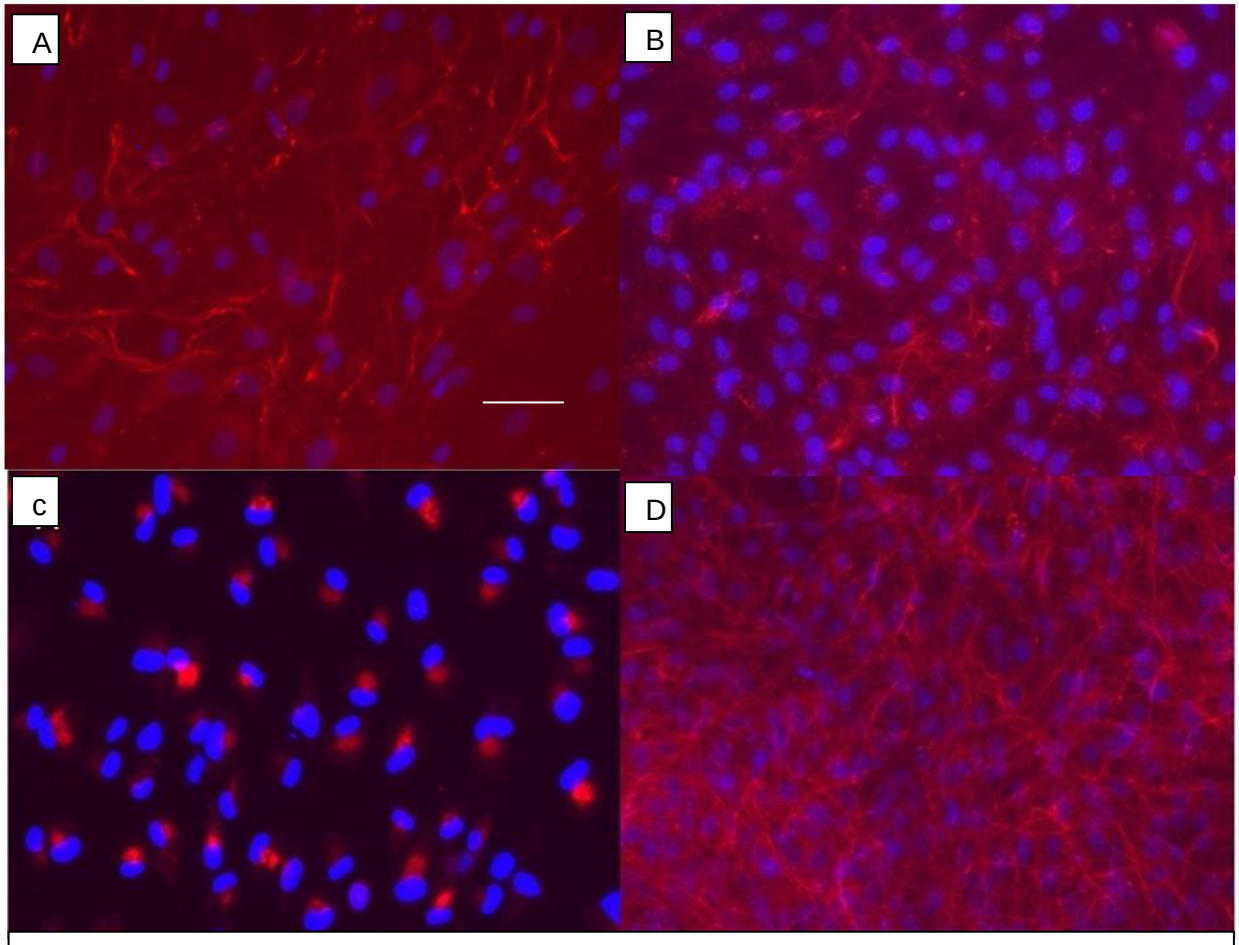


Figure 3.11: Immunofluorescence staining of Collagen XII in dermal fibroblasts of affected individuals. The IF staining demonstrated inp.Gly2786Asp (BM2) (A), p.Arg1965Cys (BM5) (B), g.75815009C>T:c.8345-8432del,p.2727-2755del(BM1')(C). Normal human dermal fibroblasts (D). Collagen fibres are shown in red signals and nucleus in blue. Scale bar in A = 100 µm

Overall, a decrease in collagen XII signals was found in mutant fibroblasts of affected individuals by visualization. In addition, an absence of collagen XII signals was found in the patient with splice donor mutation. However, degrees of variability in different staining batches were seen.

3.4.3.2 *Immunofluorescence staining for collagen XII binding partners in dermal fibroblasts of BM2 and BM5:*

Two potential collagen XII binding partners – collagen IV and tenascin X – were stained with immunofluorescence antibody in dermal fibroblasts using the previously described method. Fibroblasts of BM2 and BM5 were used. The experiment was repeated three times and five images from each slide were taken. Figure 3.12 shows the result of this staining.

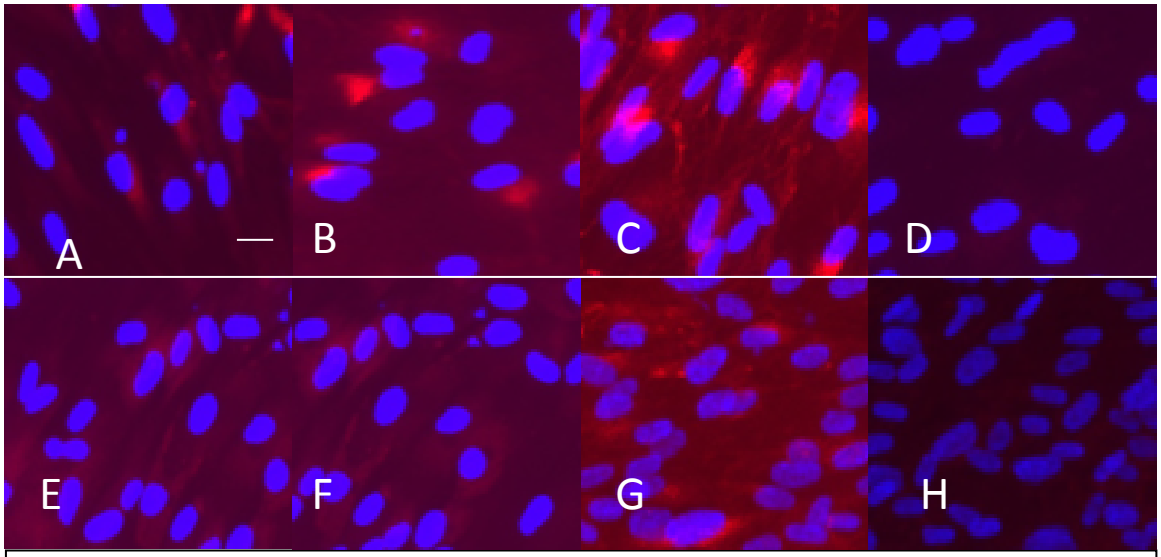


Figure 3.12: Immunofluorescence staining of Collagen XII binding partner in dermal fibroblasts of BM2 and BM5.A-D: Immunofluorescence staining of dermal fibroblasts for Tenascin X (red) in p. Arg1965Cys(BM5) (A), p. Gly2786Asp(BM2) (B), Normal control (C), Negative control (D). E-F: Immunofluorescence staining in dermal fibroblasts for collagen IVA (red) in p.Arg1965Cys (E), p.Gly2786Asp (F), Normal control (G), Negative control (H) Scale bar in A = 100 µl (Hicks *et al.*, 2014).

Tenascin X staining shows decreased signals for both mutant cells and Collagen IV staining shows less deposition of collagen IV in the extracellular matrix area. However, collagen IV signals were presented in ECM both in control and mutant individuals. The dot pattern of collagen IV in NHDFC was not visible in affected individuals.

3.4.4 *Immunoblotting of collagen XII in COL12A1 mutant individuals:*

The proteins were extracted from fibroblasts of all mutant individuals. Figure 3.13 demonstrates the results for the BM2 and BM5 individuals.

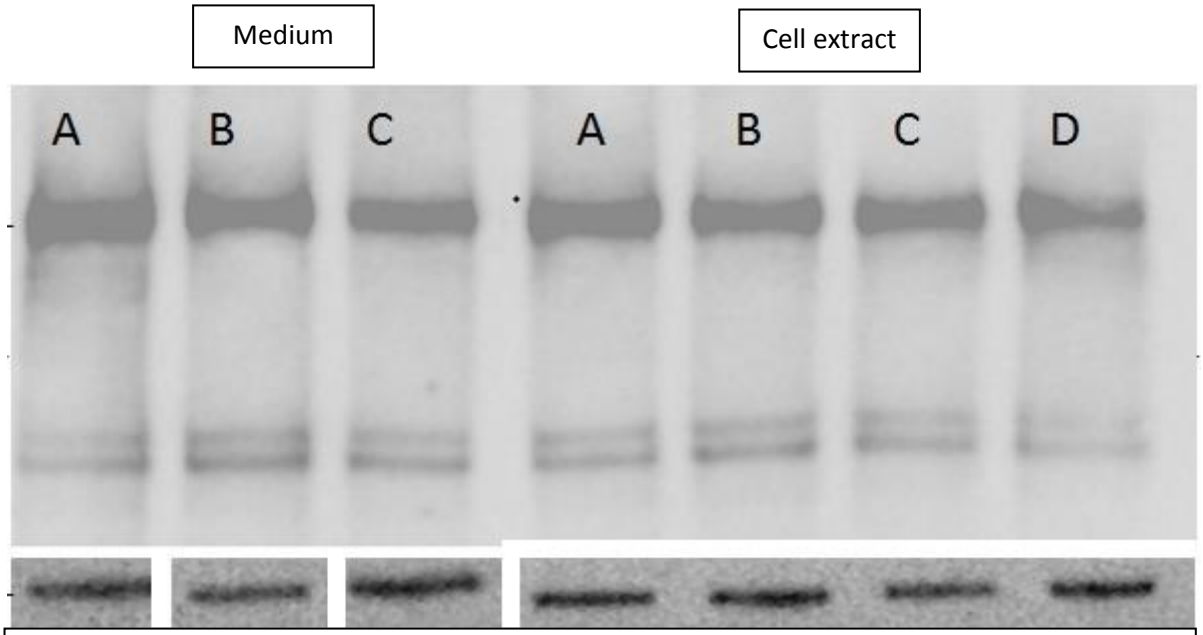


Figure 3.13: Westernblot for collagen XII in BM2 and BM5. Collagen XII westernblotting in p .Arg1965Cys (BM5) (A), p.Gly2786Asp (BM2) (B), normal control (C) and commercial control (D).The left A, B and C show protein detected in the cell culture medium. The bottom picture shows staining with Emerin as the control antibody for the loading amount. Collagen XII bands are predicted at 330 kDa and Emerin as control at 35 kDa. Images were taken by hlp immunoblotting system (Hicks *et al.*, 2014).

There was no difference between the mutant and normal cell lines with regards to the amount of protein. Also, the amount of protein which is secreted to media is the same between the mutants and normal control.

Figure 3.14 shows the result for splice donor mutation (BM1') and measurement of its westernblot bands' density.

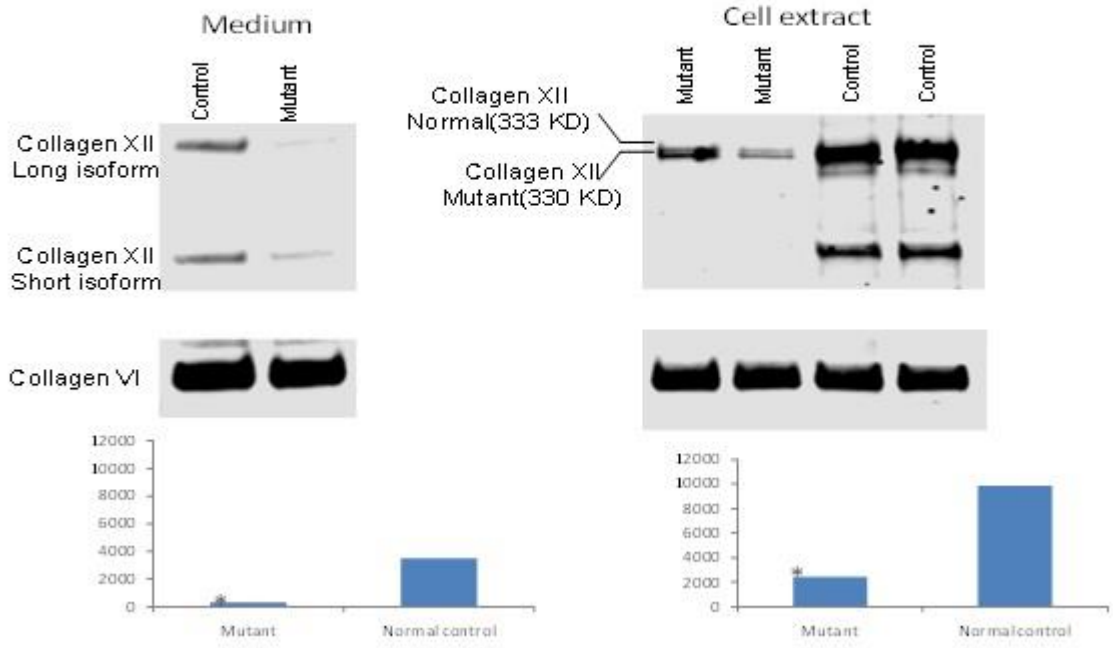


Figure 3.14.Western blot for collagen XII in BM1'. 1) Western blot for collagen XII in media and cell extract in mutant patient with g. 75815009C>T. c.8345-8432del, p.2727-2755del (BM1') and normal control. Collagen VI were used as control in both systems. The underline graph shows a significant reduction in the amount of protein in mutant cells in compare to normal controls both in medium and cell extracts. Images were taken with Odyssey system.

The splice donor mutation has a significantly lower protein amount compared to NHDFCs, also the amount of protein which is secreted to the medium is less than NHDFC in the mutant sample. Collagen XII both isoforms which translated from two transcriptional variant of collagen XII represented in extracts.

3.4.5 *Collagen XII immunofluorescence staining in normal mouse muscle:*

Normal mouse muscles (Rectus femoris biopsy) were stained for collagen XII and compared to the collagen VI pattern of mouse muscle staining (Figure 3.15) .It demonstrated that collagen XII has the same localization as collagen VI.

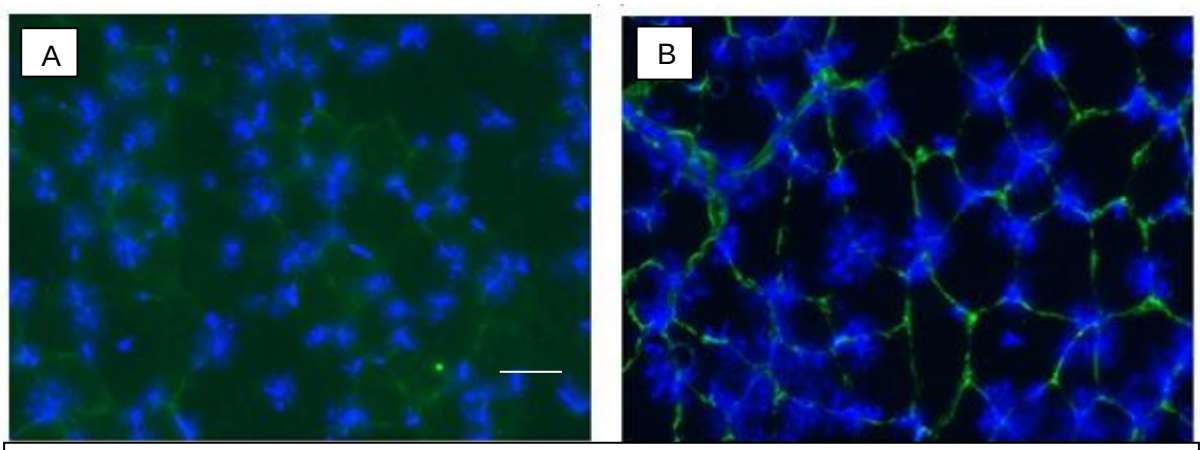


Figure 3.15: Collagen XII staining in mouse muscle. The left picture represents collagen XII in mouse muscle and the right one represents collagen VI in mouse muscle. Collagen fibres which have a basement membrane appearance are in green. Nucleus is blue with DAPI staining. Scale bar in A = 200 µm.

3.5 Discussion:

Genetic heterogeneity was described and reported in a number of patients with Bethlem-like myopathy phenotypes (Hicks *et al.*, 2008). The MRC Centre for Neuromuscular Diseases in Newcastle and other centres have reported and followed up such patients. MRC Centre for Neuromuscular Diseases estimated that about 50% of our collagen VI-related myopathy phenotype patients referred to its affiliated clinic in Newcastle are not associated with *COL6A* mutations (Hicks *et al.*, 2014).In this study, we aimed to identify novel disease-causing genes for a cohort of the Bethlem-like myopathy phenotype which do not have any disease causing mutations in their *COL6A* genes.

This cohort shows some symptoms of collagen VI-related myopathy (predominantly the milder form, Bethlem myopathy) and other symptoms of overlapping different disorders. Joint contractures, muscle biopsy abnormalities and a high creatine kinase level appeared to be the most frequent symptoms. Abnormality in muscle reflexes was not seen in any patient that ruled out the chance of being neurologically impaired. Less than 10% of patients in this cohort were non-ambulant or wheelchair-

dependent. Abnormalities in immunofluorescence staining and positive family history are the other rare symptoms. These symptoms showed that many patients suffered from a mild to moderate disorder. Moreover, for heterogenic Bethlem-like myopathy phenotypes the symptoms which represent connective tissue involvement such as joint contractures and spine abnormality have a higher frequency. Different muscle abnormalities as an indicator of neuromuscular disorder were found in these patients.

We identified *COL12A1* as a potential disease-causing gene for two patients of this cohort with their families. Later, one patient with joint hypermobility and slight muscle weakness from a cohort of undiagnosed patients was identified with a splice donor mutation in *COL12A1.* Muscle weakness appeared to be fine in all patients and none of them loosed their ability to walk. In one family of point mutation with two affected members muscle weakness improved with age in three family patients. Joint hyperlaxity and skeletal abnormalities were noted to be the most prominent and common symptoms in all patients. Very recently, mutations in *COL12A1* were also reported in two other patients with a collagen-VI/ Ehlers-Danlos myopathy symptoms overlap (Zou *et al.*, 2014). The most affected patient with nonsense mediated decay suffers from severe muscle weakness, joint hypermobility and surgically fixed scoliosis. Lipodystrophy was one new symptom that is found in one of our patients with the splice-donor mutation (BM1') of *COL12A1* and could widen the spectrum of *COL12A1* myopathy phenotype. Overall, the new cohort of *COL12A1* mutant patients demonstrated more symptoms of joint hypermobility and skeletal abnormality with less severity in muscle weakness. Even patient with lipodystrophy sharing those symptoms with other affected individuals. These unifying symptoms could be beneficial for clinicians to include *COL12A1* in the list of differential diagnosis.

The collagen XII molecule is a member of FACIT collagens which consist of collagen IV, IX, XII, XIV, XV and XIX as other members. FACIT collagens are a recently identified class of collagen molecules. They are unable to form fibres by themselves but by means of attaching to other collagen fibres they may control the

architecture of interstitial fibrils. These interstitial fibrils build up the insoluble framework common to each matrix type (Halper and Kjaer, 2014). Collagen XII is an extracellular matrix protein (ECM) and like other collagens has a specific site for glycose aminoglycans such as heparin to bind. RNA expression analysis revealed that *COL12A1* is expressed as two homotrimer protein variants. The larger variant contains 330 amino acids and has a more restricted expression pattern similar to tenascin X. The small variant contains 220 amino acids. Both variants are comprised of a different fibronectin type (FN3) and von Willebrand A domain (VWA) which is similar to other collagen molecules. Eight more FN3 domains and two more VWA domains were discovered in the larger variant. Also like the other collagen family, the collagen XII molecule has collagenous domains which are unique to this molecule. The collagenous domains are near to the COOH terminal. Collagenous domains1 and 2 (COL1, COL2) are short but collagenous domain 3 (COL3) is large specifically in the larger variant, and reveals a complex structure with a high affinity for molecular interactions. Several studies suggest an association between the collagen XII and collagen I striated fibre (Koch *et al.*, 1995; Agarwal *et al.*, 2012). Also, biochemical studies revealed that the large variant of collagen XII has a higher affinity to attach to heparin. As the collagen XII-heparin biochemical complex with the large variant does not detach in a higher salt concentration, a shock absorber role for collagen XII among other FACIT collagens is suggested (Trachslin *et al.*, 1999). All the variants we found in our study affect the larger *COL12A1* variant.

The variants NM004370: c.5893T>C: p.Arg1965Cys and NM004370: c.8357A>G: p.Gly2786Asp both are in the FN3 domain of the protein in the non-collagenous region. The variants in BM1' and BM2 do not exist in the ExAc control database. Also, analysis with Polyphen2program predicted these changes to be pathogenic. Other *in silico* analysis revealed that these changes are located in highly conserved regions among species. Fibronectin type 3 domains are the largest domains in the fibronectin protein and are also represented in other extracellular matrix proteins. They are evolutionarily conserved domains and exhibit the sites of interactions with other molecules. These sites are mostly mapped with short repeats of amino acids such as Asp-Gly-Arg (RGD). None of the mutations we found interrupted the short

stretch of RGDs. However, NM004370: c.5893T>C:pArg1965Cys by adding one cysteine amino acid interrupted disulphide bonds which are important in the formation of the secondary structure of the protein. An aberrant structure of the protein could result in trapping the protein in the endoplasmic reticulum or Golgi apparatus. Further experiments of protein trapping for BM2 and BM5 were done by endoplasmic reticulum imaging on skin biopsies of these affected individuals (Hicks *et al.*, 2014). Due to unavailability of skin biopsies for BM1' the work did not repeated on these patient. Afterwards, the unfolded protein response pathway will be activated to digest and eliminate the protein. The unfolded protein response pathway has been suggested to be one of the mechanisms underlying neuromuscular disorders. Further evidence for unfolded protein response pathway activation for BM2 and BM5 was discussed in the article by Hicks et al (Hicks *et al.*, 2014).

In that article it has been shown that Golgi apparatus was enlarged in skin biopsy samples which were taken from the patient with the NM004370: c.5893T>C: pArg1965Cys (BM5) mutation by electron microscopy. Also, the unfolded protein response pathway is upregulated in the BM5 mutation by RNA studies (Hicks *et al.*, 2014).

Another possible mechanism for the NM004370: c.8357A>G: p.Gly2786Asp mutation was suggested*. In silico* analysis revealed that amino acid substitution in this patient occurred in a highly conserved Gly-X-Y motif. These repeats are most important in collagen structure and mutations in them are reported to cause different disorders. Also, substitutions of glycine residues in the Gly–X–Y motifs of the N-terminal collagenous triple-helical domain of collagen VI are reported to be one of the important causes of collagen VI-related myopathy (Lamande *et al.*, 2002). This type of mutation in collagen VI patients acts in a dominant-negative fashion. We have seen the same fashion in a collagen XII mutant patient. In collagen VI patients mutated α-chain subunits can become incorporated into collagen VI monomers. Evidence of an extra ultrastructurally visible kink in the completed triple-helical domain has been reported in these patients (Hicks *et al.*, 2009b).The molecular and

clinical consequences of autosomal-dominant substitutions in Gly-X-Y motifs are variable and depend on the exact sequence context in which they occur (Lamande *et al.*, 2002).

Glycine substitutions might, therefore, be seen in dominant pedigrees affected by typical Bethlem myopathy, but also occur as *de novo* mutations in patients with collagen VI-related myopathy phenotypes in the Ullrich CMD or intermediate range (Camacho Vanegas *et al.*, 2001). We observed a reduction in the immunofluorescence signals of collagen XII for patient with glycine residue substitution. This pattern could be due to inability of the protein to interact with other ECM components and the consequent instability. However, there are limitations in structural evaluation of protein with immunofluorescence staining.

The first attempt to IF stain patient cells for collagen XII with an in-house protocol (Hicks *et al.*, 2008) did not reveal any differences between normal and mutant cells. The optimized protocol which has an antigen-sensitive step which is permeabilize cell membrane with cloridric acid solution and was used before for collagen IV staining was used afterwards, which reveals a reduction in collagen XII signals (Shen *et al.*, 2014). Immunofluorescence staining could not show whether this difference is a result of a quantitative reduction in the protein or if the protein is not assembled correctly and could not collaborate in the extracellular matrix structure (Jimenez-Mallebrera *et al.*, 2006). Westernblotting for this patient also showed the quantitative amount of the protein for the two-missense mutations is not reduced and the amount of the protein which is secreted to the extracellular matrix structure is similar in mutant and normal cell lines. These findings in conjunction with IF staining results suggest that while misfolded protein could assemble correctly it could not contribute to ECM structure. However, the splice donor mutation shows a reduction in the amount of protein secreted to the medium which reflects a defect in intracellular protein assembly. This defect could cause the accumulation of the protein in the endoplasmic reticulum. Endoplasmic reticulum degradation pathway (ERAD) might be activated. One possible future experiment is to inhibit ERAD

pathway by drugs such as eeyarestatin and check whether IF staining could show signal increase due to release of the misfolded protein from endoplasmic reticulum. Fibroblasts of control and affected individuals will be treated with 1µl of eeyarestatin and then IF staining for collagen XII will be done for eeyarestatin treated and untreated cells for mutant and control individuals. We expect that the inhibition of endoplasmic reticulum degradation pathway with eeyarestatin lead to presentation of more collagen XII signals in mutant samples but many of these collagen XII fibrils might not be capable of assembling into extracellular matrix.

So far, one other individual with *COL12A1* mutation was reported by Punetha et al in 2017 (Punetha *et al.*, 2017).They describe an 8-year-old Polish girl who has symptoms of profound hypotonia and joint hyperlaxity at birth. Her mother during this pregnancy suffered from oligohydramnios and intrauterine growth retardation.They identified a novel, potentially pathogenic heterozygous missense *COL12A1* c.8329G>C (p.Gly2777Arg) variant using a targeted sequencing panel. Patient fibroblast studies confirmed intracellular retention of the collagen XII protein, consistent with a dominant-negative mutation. They reported that the patient showed a more intermediate phenotype, thus could expands the phenotypic spectrum for *COL12A1* mutant affected individual. They suggested the patient presented much of overlapping features of Ehlers Danlos syndrom and Bethlem myopathy (Punetha *et al.*, 2017).

The above case is also another example of *COL12A1* affected individuals who represent with mild to moderate muscular symptoms and connective tissue symptoms as hyperlaxity. The new patient together with other reports of*COL12A1* affected individuals proved the phenotypic diversity of these group of mutant individuals. However, so far symptoms such as joint hyperlaxity and mild to moderate muscle weakness were found to be common in all cases. One rare symptom of lypodystrophy was found in one individual of our cohort (BM1`) but as the number of *COL12A1* affected individuals are still few, we need more report of *COL12A1* mutations in order to be able to describe unique symptoms for this entity (Hicks *et al.*, 2014; Zou *et al.*, 2014; Punetha *et al.*, 2017).

As mentioned above, one possible collagen-XII role is to connect different extracellular matrix molecules to each other or to attach them to the main extracellular matrix collagen fibrils such as collagen I and II. It is observed that collagen XII along with other matrix molecules such as fibronectin, tenascin and vimentin could change the extracellular matrix deformability. Immunofluorescence staining for other matrix molecules which might connect to collagen XII as binding partners shows a different pattern compared to normal controls. A reduction in both collagen IV and tenascin signals was seen in mutant fibroblasts. This phenomenon could explain the effect of mutant collagen XII molecules in patients' extracellular matrix samples.

Localization studies suggest that collagen XII protein variants could work as interfibrillar attachments but this function could not be described in detail (Koch *et al.*, 1995). It would be reasonable to predict that, as a fibril-associated molecule they might either cross-link or separate collagen fibrils while determining or maintaining interfibrillar distances. This function appears unlikely for collagen XII variants because they appear at all collagen distance. Such molecules might also be predicted to secure the collagen fibril to the elastic network or the type VI collagen network. While interactions of collagen XII variants with type VI collagen are not excluded by these studies, there is no obvious colocalization of collagen XII variants with type VI. Preliminary mouse muscle staining for collagen XII showed a localization pattern similar to collagen VI. However, none of the patient muscle sample were available for collagen XII staining. Lack of these samples is one of our study limitation which makes it difficult to comment on direct effect of collagen XII molecular abnormality in muscle samples.

While the first part of our study revealed a possible abnormal ECM structure for patients with *COL12A1*mutations, a broader research on possible effect of these mutations on extracellular matrix structure was done in the next chapter. Our findings emphasize the role of *COL12A1* as a disease-causing gene in patients with Bethlem-like myopathy symptoms. *In vitro* molecular studies also support the idea of an

abnormal structure of the mutant proteins. However, the exact role of collagen XII in the pathogenesis of the disease remains to be discovered.

Chapter 4 Extracellular matrix hierarchical structure in *COL12A1* mutant individuals

4.1 Introduction: Collagen XII molecular structure, assembly, and role in extracellular matrix

Extracellular matrix (ECM) molecules are classified as collagens, elastin, proteoglycans and other non-collagenous structural/cell interaction proteins. Distinctive repetitive modules are common structural features among ECM molecules, which could resemble the functional domain in non-ECM proteins (Allamand *et al.*, 2010). Collagen molecules are the most abundant form of protein in the extracellular matrix and were produced by ECM fibroblasts. These molecules consist of three polypeptide chains which are called α chains (Zulian *et al.*, 2014). The unique protein domain of this molecule, a collagenous domain, is made up from intertwined α chains in a rod-like shape. GLY-X-Y motifs appear in all collagen molecules. GLY is glycine X and Y in these motifs are mainly proline and hydroxyproline (Halper and Kjaer, 2014). This sequence is required for the correct assembly of collagen molecules in which Glycyl residues form the centre of the triple helix, while folding and stabilization of the triple helix is dependent on amino-acid residues. Collagen molecules are not only important in ECM and connective tissue structures but they also have a signalling role. Two main groups of collagens are fibrillar and non-fibrillar. Fibrillar collagens are types I, II, III, V, XI. Non-fibrillar collagens are comprised of different types of collagens such as FACITs (Fibril Associated Collagens with Interrupted Triple Helices), short chain, basement membrane, multiplexin (Multiple Triple Helix domains with Interruptions), MACIT (Membrane Associated Collagens with Interrupted Triple Helices), and others.

So far, 28 different types of collagens have been discovered. A great level of diversity both in α chain structure and supramolecular aggregates is seen in different types of collagens. All fibrillar collagens are composed of ten different polypeptide α chains which are a1(I), a2(I), a1(II), a1(III), a1(V), a2(V), a3(V), a1(XI), a2(XI) and a3 (XI) (Young *et al.*, 2002). These chains are descended from a common ancestor coded by a 45 to 54 nucleotide gene. The product of this gene is a 15 to 18 amino acid long polypeptide which is comprised of repeating motifs of GLY-X-Y residues. During evolution, this ancient gene, through successive duplications and mutations

has grown into a family of large, related, and structurally interrupted genes which encode long products of 1200-1300 residues. However, the GLY-X-Y motifs are still preserved and a hallmark of the collagen family. While the nucleotide sequence of different collagen genes has been deciphered, the nucleotide/amino acid correspondence is not entirely clear for all collagen molecules (Mienaltowski and Birk, 2014b). This is mainly due to extensive post-translational modifications in the collagen molecule. Hydroxylation of lysine and proline residues is an essential step of post-translational modification and critical for collagen structure (Wenstrup *et al.*, 2011).

A great number of genetic hereditary diseases in humans are caused by collagen mutations, which shows the prominent role of collagen molecules in biomechanical and biochemical properties of different tissues. Some other variable and important post-translational modifications include oxidation and glycosylation. Evaluation of the primary structure of collagens reveals other features such as the distribution of some amino acids (lysine, glutamine, and arginine) which show a periodicity which reflects the end of an exon or clustering of polar and hydrophobic residues that repeats periodically in every 234 amino acids (Chen *et al.*, 2014).

The secondary structure of fibrillar collagen is an α-helix. It is made up of the folding of polypeptide α-chains with an axial residue-to-residue spacing of 0.286 nm and angular separation of 108°. This structure is highly dependent on the steric repulsion between proline and hydroxyproline in the X and Y positions. This proline-based backbone results in a side chain of amino acids being exposed to the outside. Because of the geometry of α-helixes, which turn every three residues, and the third place position of glycine in the GLY-X-Y repeat, the surface of α-helixes contain a glycine row (Gordon *et al.*, 1987). Point mutations which appear in the X and Y positions might be tolerated but mutations in glycine residues always lead to a range of congenital disorders of connective tissue, because they clearly interrupt the α-helix structure and any further intercellular assembly (Lamande *et al.*, 2002). Collagen fibrils are either homo- or heterotrimers of α-helixes. This depends on

collagen and tissue type. Collagen I is always a heterotrimer of a1(I)2 + a2(I).The triple helix is a rod-like, stiff but flexible, structure which is 1.5 nm wide and 300 nm long with a globular domain at each end (Izu *et al.*, 2011b). This form is called procollagen which is excreted into the extracellular space for further assembly. In the extracellular space, the terminal globular domains are split to form collagen. This is a highly reactive molecule which very quickly becomes involved in the formation of fibrils. Fibrillogenesis is the generation of a large supramolecular structure from collagens. This structure first forms through polar, hydrophobic, and other non-covalent bonds. Then they are stabilized by covalent bonds(Harris *et al.*, 2005).

Many different techniques have been used to investigate collagen structure. Transmission Electron Microscopy (TEM) images have provided most of the information about the collagen supramolecular structure, while X-ray diffraction systems have helped to understand the lateral fibril structure. However, X-ray ability to demonstrate all the changes is limited. This approach is appropriate for simultaneously revealing fibril-bound material, surface features such as the cross-banding, and some details of the inner structure of the collagen fibril, while avoiding the artefacts usually caused by fixation, dehydration or sectioning (Harris and Reiber, 2007).

FACITs as the main focus of this chapter include collagen types IX, XII, XIV. These types of collagens do not form quarter-staggered fibrils by themselves because their α-helix is a mixture of globular and collagenous domains, thus they cannot form fibrils so they are non-fibrillar collagens (Starborg *et al.*, 2008b). The structure of these molecules can be divided into three functional parts. One part which consists of one or two functional domains serve for the attachment of the superstructure of the molecule to the fibrils. The second part has a triple helical domain, working as a rigid arm to project the third part out of the fibril. The third part and its functional domains may serve as a region to interact with ECM components and cells. The structure of the FACIT protein is comprised of triple helical domain (collagenous domain) and interrupted non-triple helical domains (non-collagenous domain).Type IX collagen is the best identified example of FACITs. This molecule is the first FACIT

that was discovered. This triple helical molecule is attached to ECM containing fibrillar type II collagen, such as the vitreous body of the eye and hyaline cartilage. This attachment is served by covalent cross-links. The role of collagen IX in this interaction remains to be elucidated completely. However, studies on chicken cartilage suggest collagen IX might help the attachment of large glycosaminoglycan side chains to collagen fibrils. In the ECM, collagen IX might be the means of attachment to fibrils. Absence of collagen IX in many connective tissues has revealed a need for an alternative molecule in other tissues with a similar functional role. Gordon et al (1987) successfully searched for such molecules (Gordon *et al.*, 1987).

Collagen XII was discovered from chick tendon fibroblasts in 1987 during research for novel collagenous sequences (Gordon *et al.*, 1987). Collagen XII cDNA was highly similar to collagen IX, which is characterized above as a FACIT collagen. Dublet and vander Rest isolated collagenous pepsin-resistant fragments from chick tendons, whose amino acid sequences matched the published collagen XII cDNA, in the 1989 (Dublet *et al.*, 1989). In 1989, the intact protein of collagen XII was characterized as a homotrimer of 220-kDa subunits, with disulphide-bonded 190-kDa non-collagenous domains linked to a short C-terminal collagen helix by Doublet and colleagues. The newly extracted molecules from the tendon, collagen XII, are cross-shaped with three 60 nm long arms and a thinner tail 75 nm in length (Gordon *et al.*, 1989).In 1991, Yamagata et al. published the full-length chick collagen XII cDNA which encoded a subunit of 340kDa (Koch *et al.*, 1992). Larger molecular species of collagen XII which were purified from chick fibroblasts and a human cell line confirmed that two isoform of collagen XII is existed (Lunstrum *et al.*, 1991; Koch *et al.*, 1992). Peptide sequencing has shown these larger molecules are also collagen XII. In fact, large variants of collagen XII have non-collagenous arms of more than 300 kD and 90 nm in length (Koch *et al.*, 1992). Alternative splicing is responsible for large and small variants of collagen XII (Koch *et al.*, 1995). In 1991, a molecule with a distinct sequence and similar structure to the small collagen XII variant was discovered (Galetta *et al.*, 1991). This newly discovered molecule was named

collagen XIV. These novel molecules became the main members of the fibril-associated collagens with an interrupted triple helix (FACITs).

Later, the complete primary structure of an α1 chain of type XII collagen was identified. This molecule was extracted from chicken embryonic fibroblasts. This structure combines the findings of short separate domains into a large 3410 amino acid structure (340 kD). The extracted chicken collagen type XII sequence starts at the amino terminus with one unit of the type III motif of fibronectin, then one unit homologous to the von Willebrand factor A domain, which is followed by another fibronectin type III domain, a second von Willebrand factor A domain, six domains of type III and a third von Willebrand factor A domain. After this, ten consecutive units of fibronectin type III motif and a fourth von Willebrand factor A domain are placed. Before the start of the collagenous domain, one non-collagenous domain similar to the non-collagenous domain type four of collagen IX is placed. At the end of the molecule are two short collagenous domains. A motif of Arg-Gly-Asp as potential cell adhesive recognition sequence is present in a terminal part of one collagenous domain in a hydrophilic region. Different antibodies have been raised to type XII collagens. These antibodies can recognize both variants of collagen XII and also non-specific bands of different size (Koch *et al.*, 1995; Gordon *et al.*, 1996). Figure 4.1 shows a schematic picture of collagen XII protein.

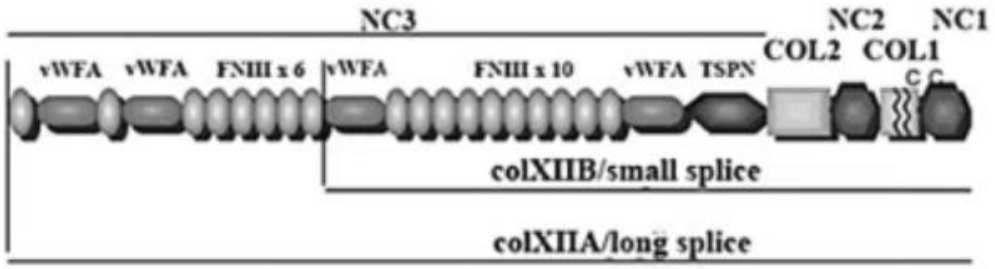


Figure 4.1.Schematic picture of collagen XII. Collagen XII protein short and long isoform are represented in this picture.

Antibody-specific studies have shown collagen XII is widely expressed in mesenchymal tissues containing collagen I such as in developing bone, ligaments, tendons, fibrocartilage, smooth muscle and skin (Anderson *et al.*, 2000). It is also

found in tissues with the collagen I matrix of foetal articular cartilage, mostly in its superficial layers (Williams *et al.*, 1992). In adults, the expression of collagen XII is more restricted to dense connective tissues such as the periodontal ligament (Karimbux *et al.*, 1992), dermis around hair follicles and cornea of the eye (Anderson *et al.*, 2000). Fibroblasts are the primary source for the secretion of collagen XII in adults and in embryo mesenchymal cells are the sites for collagen XII secretion. Large and small variants of collagen XII are expressed differently(Anderson *et al.*, 2000).However, the gene expression mechanism and function of different collagen XII variants are not clearly understood. Gene expression might be upregulated during osteogenesis and some cancer progressions, according to different studies (Tzortzaki *et al.*, 2006; Karagiannis *et al.*, 2012). It has been suggested that tensile strain by acting on cells regulates collagen XII expression. There is a promoter responsible for tensile strain in *COL12A1* in the chick's first exon. Also, another promoter for C-Jun was identified in the *APO1* promoter for *COL12A1*(Arai *et al.*, 2008). Localization of collagen XII in basement membrane has been reported, but it's role is unknown (Farhat *et al.*, 1979). Investigations showed *in vivo* expression of collagen XII induced by chronic loading (Karimbux *et al.*, 1992). According to these findings, static versus strain forces regulate *COL12A1* gene expression (Kania *et al.*, 1999; Trachslin *et al.*, 1999).

Collagen XII is important in extracellular matrix assembly. First, immunogold labelling studies by Koch et al showed placement of collagen XII on large collagen I ECM fibrils. Second, in this study it was shown that decoration of collagen XII could be reconstituted *in vitro* by adding collagen XII on to large collagen I fibril monomers. In this study, it was also shown that in the absence of collagen XII, collagen I monomers are thicker. Collagen XII also interacts with neighbouring compartments in ECM (Koch *et al.*, 1995; Veit *et al.*, 2006). For example, the non-collagenous type III domain of the molecule which is protruding from the fibrillar structure is bound to tenascin-X, another ECM component. Immunoelectron microscopy studies showed collagen XII was attached to decorin, fibromodulin, and cartilage oligomeric matrix protein molecular components as well (Bateman *et al.*, 2009). Collagen XII with this binding partner could form a bridging network to absorb sheer stress forces on

collagen fibrils and attach them to each other (Wessel *et al.*, 1997; Trachslin *et al.*, 1999).

Apart from diverse studies that showed a possible role for collagen XII in ECM assembly and characteristics, collagen XII deficient animal model, repeated some clinical features of mutant *COL12A1* individuals (BM2, BM5 and BM1') who we described in previous chapter. *COL12A1*-knockout mice suffer from muscle weakness and joint contractures which could be the result of increased elasticity in muscle tissues and lack of strain forces. Also in these mice, the histologic pattern of bone meshwork is siginficantly disorganized which is represented in mouse kyphoscoliosis and shorter long bones (Zou *et al.*, 2014). Formation of osteocyte nodules in the osteoblast culture of a *COL12A1*$^{-/-}$ mouse is delayed, and the polarity of cells is decreased. It seems that the collagen XII protein is important for proper osteoblast/ osteocyte differentiation, either through direct contact or a signalling pathway. Integrinα1β1 is a potential candidate signal molecule for collagen XII acting in the osteocyte differentiation pathway (Arai *et al.*, 2008; Izu *et al.*, 2011b). However, it might not act similarly in all creatures. The integrin α1$^{-/-}$ mouse phenotype does not resemble the collagen XII null mouse, which suggests a different role for these molecules (Bolduc *et al.*, 2014).

We identified missense and splice donor*COL12A1* mutations that are responsible for Bethlem-like myopathy phenotypes (Hicks *et al.*, 2014). Bethlem myopathy as a collagen-VI-related myopathy as previously described is a muscle/connective tissue disorder which could have overlap with other connective tissue disorders such as Ehlers-Danlos syndrome (Wessel *et al.*, 1997). Around 50% of collagen VI-related myopathy patients have a normal collagen VI level and no mutation in the collagen VI genes (Hicks *et al.*, 2008). Overall, the identified patients suffer from muscle weakness, joint hyperlaxity, skin tags and skeletal problems such as scoliosis (Bonnemann, 2011).

One of the families we identified with a point mutation has structurally abrupt disulphide bands (BM5) and in the other family a Glycine residue substitutes in the Gly-X-Y motif (BM2). These patient symptoms improve with age. The last family with splice donor mutation shows more joint hyperlaxity. Also, lypodystrophy was seen in the patient which is a new symptom in the collagen XII myopathy cohort (BM1'). Reduced collagen XII signals were present in ECM immunofluorescence staining of patients while immunoblotting did not show any reduction in the protein secreted to ECM in BM2 and BM5 family. This demonstrates that misfolded protein was detected but could not assemble into ECM structure. Two other affected *COL12A1*individuals which reported parallel to our cases show the same symptoms with a change in the expression level of the collagen XII protein (Bolduc *et al.*, 2014; Hicks *et al.*, 2014; Punetha *et al.*, 2017). These findings support the pathogenic nature of mutations and their consequent effect on connective tissue. However, the link between human connective tissue pathologies and molecular interactions of collagen XII in the ECM structure is unknown.

Based on previous findings, we aim to identify the role of collagen XII in the ECM ultrastructure. First the skin biopsy of one affected individuals (BM5) was taken and compared with healthy donors. Then fibrillar collagen structures from affected individuals (BM5 and BM1') and healthy donors were evaluated both in cultured samples of fibroblasts and *in vitro* assembled samples. We hypothesized that misfolded collagen XII may lead to larger collagen fibrils diameter during collagen fibrillogenesis.

4.2 Aim:

In this chapter we aim to:

Investigate collagen fibres structure from *COL12A1* mutant individuals and compare it to healthy donors.

Measure collagen fibrils diameter in ECM structure surrounded fibroblasts of *COL12A1* mutant individuals and compare it to healthy donors.

Measure collagen fibrils diameter in *in vitro* assembled collagen fibrils of *COL12A1* mutant individuals and compare it to healthy donors.

4.3 Evaluation of collagen fibrils diameter in extracellular matrix of *COL12A1* mutant individuals:

4.3.1 Electron microscopy evaluation of skin biopsies from BM5:

4.3.1.1 *Individuals:*

Skin biopsies were taken from patients with the p.Arg1965Cys mutation (BM5) from keloid scars in Newcastle University affiliated hospitals. The preparation for electron microscopy was performed by the staff of electron microscopy unit in Newcastle University. Then the samples were compared with a skin biopsy of healthy donors. Healthy donor was matched in age and ethnicity with mutant individuals. Healthy donor was one of control samples which is kept in electron microscopy units. The examiners were blind about the nature of the samples.

4.3.1.2 *Electron microscopy findings:*

The findings were based on evaluation at the ultrastructural level by TEM. Cross-sectional images show a dense packaging of collagen fibrils in the nodules in control samples. In patient samples regions with relatively loose packing were found as well. Also, a variable size and shape in the collagen fibres was seen (Figure 4.1).This variation could be a result of difference in collagen I fibrils diameter (collagen fibres in ECM of skin are mainly composed of collagen I fibrils). Previous findings as described in this chapter introduction, suggested collagen XII as a binding partner of collagen I could affect collagen I fibrils diameter and length during collagen I fibrils assembly (Agarwal *et al.*, 2012). However should bear in mind that biopsies were taken from kloid scar in BM5 patient and the abnormal findings could be just common characteristics of collagen fibres in kloid scars (Moinzadeh *et al.*, 2013). This issue will be discussed in discussion later .This variation in longitudinal sections show a disruption in collagen fibres longitudinally and laterally in patient samples. However, in the control subjects the longitudinal collagen fibres were continued (Figure 4.2). Evaluation of areas with fibroblasts in the extracellular matrix showed a dilated rough endoplasmic reticulum in the affected individuals. Also, vascular membrane thickening was found in the mutant samples by visualization (Figure 4.3). Figure 4.2, 4.3 and 4.4 demonstrate these findings.

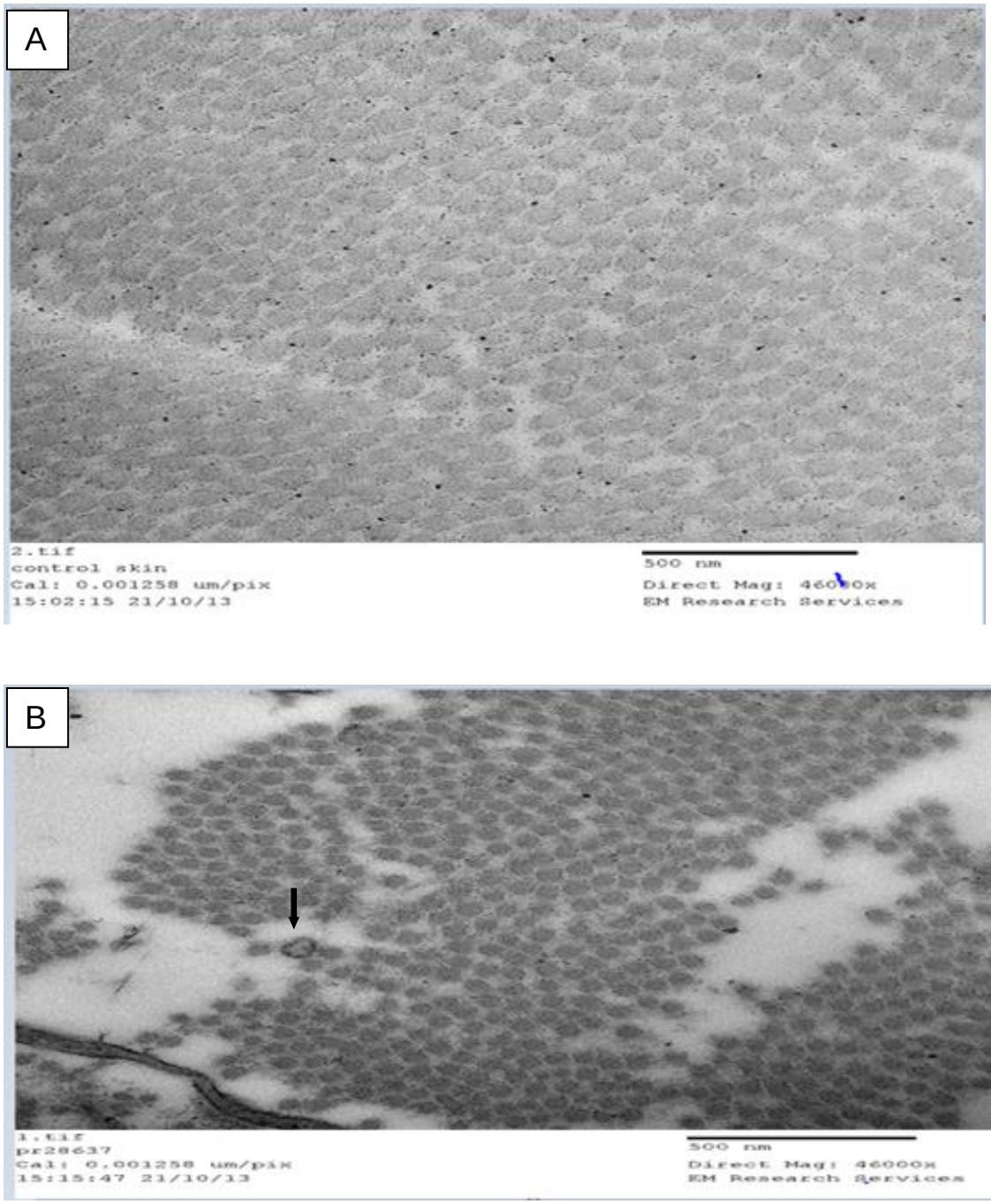


Figure 4.2. Cross-sectional images of collagen fibres. Cross-sectional images of collagen fibres in the extracellular matrix from healthy donors (A) and p.Arg1965Cys (B) skin biopsies. Black arrows indicate abnormal collagen fibre with a prominent layer in periphery of the fibre.

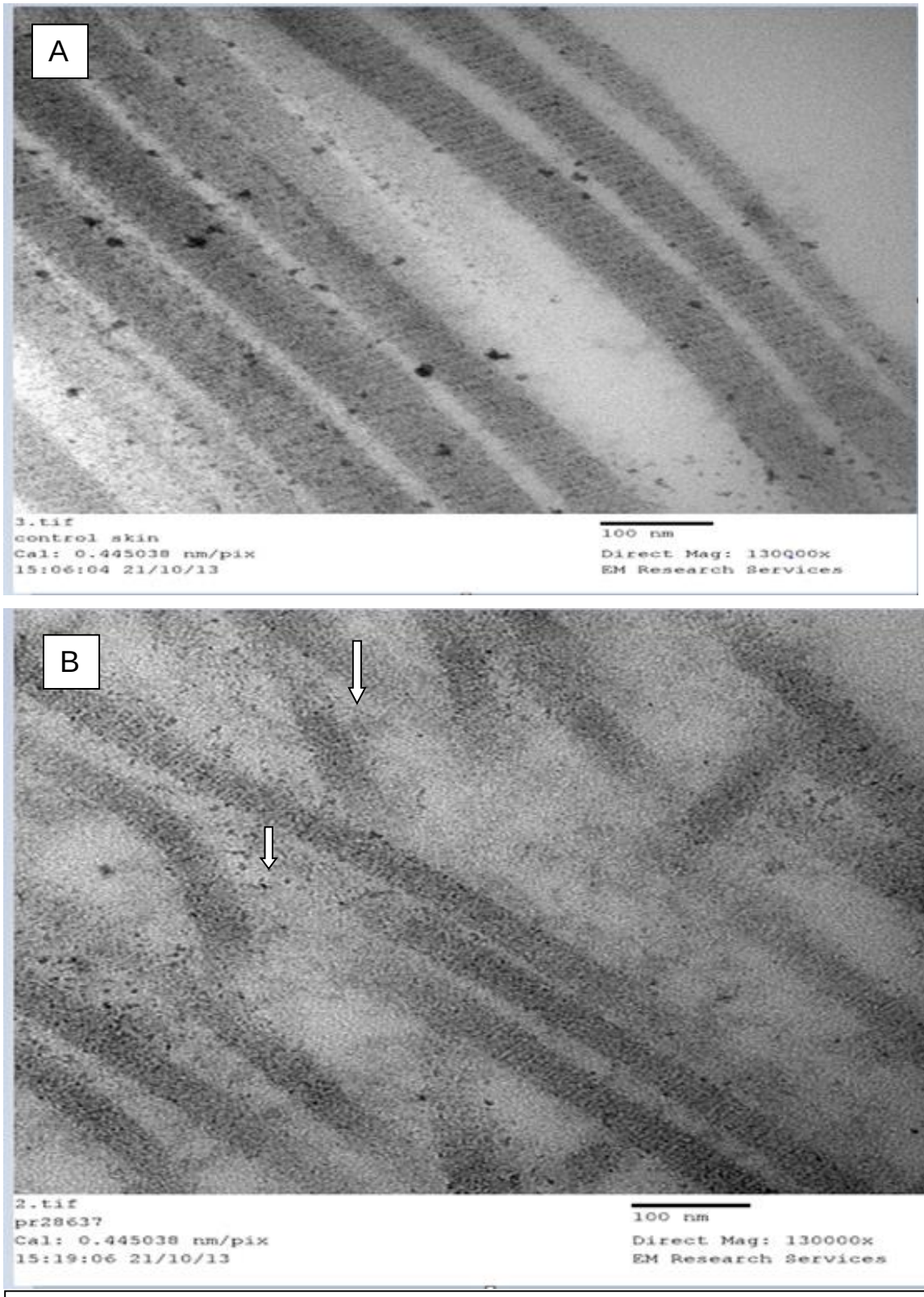
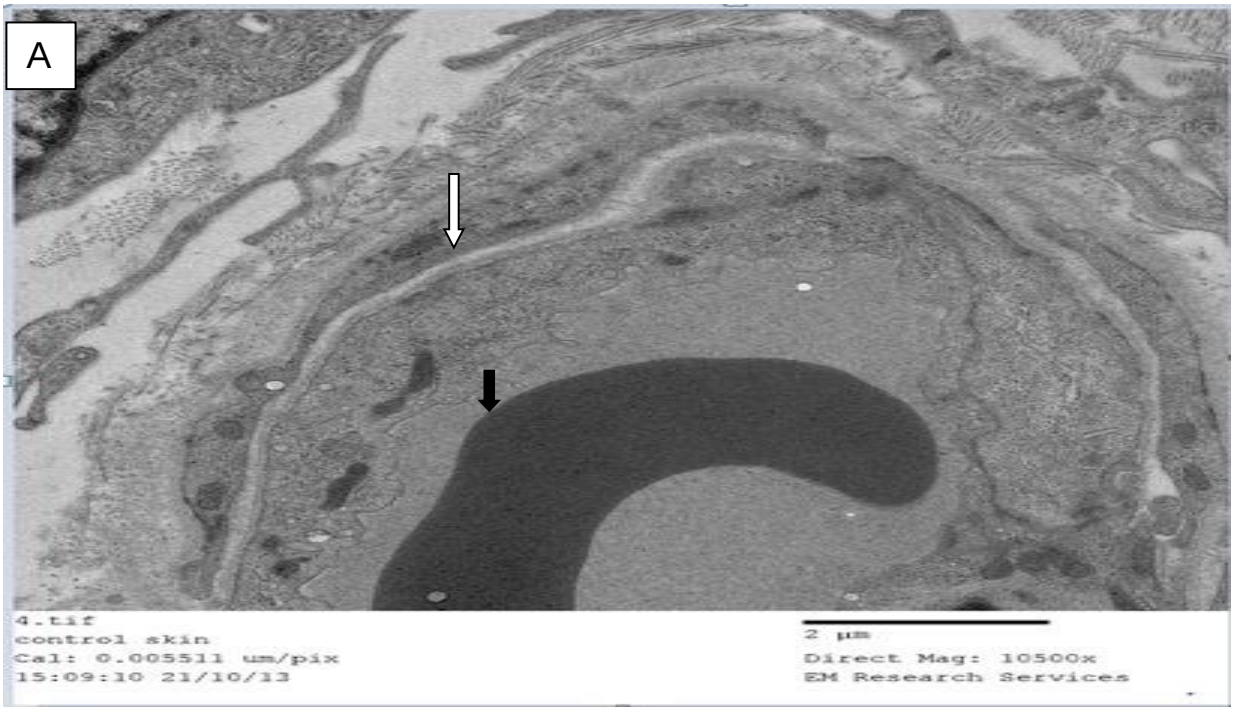


Figure 4.3.Images of longitudinal fibres. Longitudinal sections of collagen fibres in the extracellular matrix in healthy donor (A) and p.Arg1965Cys mutant cell lines (B) from skin biopsies. White arrows demonstrates sites of breaks in mutant collagen fibres.



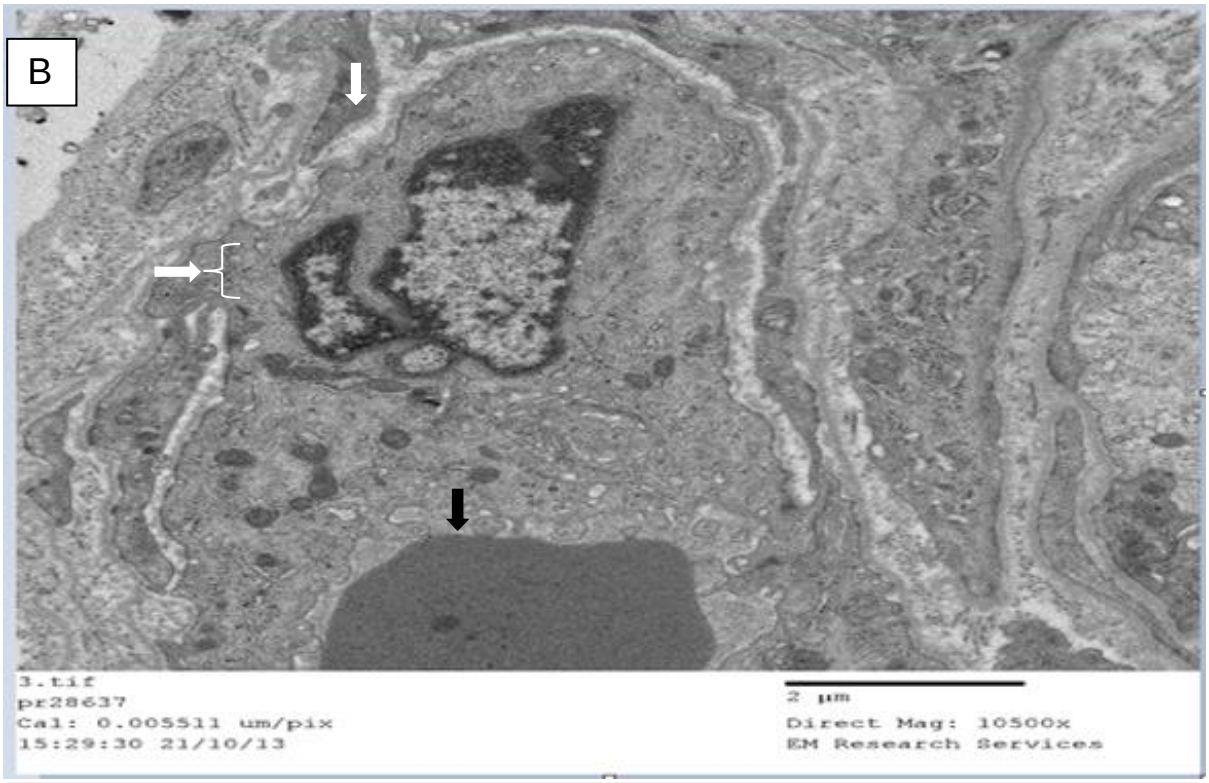


Figure 4.4.Images of vessel wall. Vessel wall of healthy donor (A) and p.Arg1965Cys skin biopsy (B). Black arrow shows red blood cells and white vertical arrow points to vessel wall thickening. White horizontal arrow points to the location of a discontinuity of vessel wall in mutant samples.

4.3.2 *Electron microscopy evaluation of collagen fibrils in ECM from BM1':*

Fibroblasts of cells with mutations from splice donor mutant individual (BM1') were cultured, and as previously described in material and methods section prepared for electron microscopy. Fibroblasts were left attached to the plastic of the culture dish and their surrounding ECM, fixed into resin and sectioned with a diamond knife. Under electron microscopy collagen fibrils were identified. These tubular structures normally appeared near fibroblasts cells. Then collagen XII molecules, which had been stained with collagen XII antibody (provided by Prof Manuel Koch) and secondary immunogold anti-rabbit antibody, appeared as single black dots on collagen fibrils. Using the silver enhancement kit for immunogold staining led to some islets of collagen XII antibody and protein conglomeration on collagen I fibrils. These artefacts were not taken into account during evaluation. Collagen XII molecules were presented in both affected individuals and healthy donors. According to previous studies, the absence of collagen XII in the *in vitro* environment for making collagen fibrils leads to an increase in collagen fibril diameter. The aim of this experiment was to assess the effect of mutant collagen XII samples on the collagen fibril diameter. After identifying locations with collagen fibrils three images were taken from each location from mutant and normal samples. In each experiment at least two locations were identified and experiments were repeated three times. In each independent image collagen monofibril diameters were measured from ten different locations horizontally. The distance between these locations was kept as 500nm. Analysis was performed using ImageJ analyser. Then unpaired t-test as used to compare measurements with the normal control, with significance set at 5%. Excel was used for analysis. This experiment showed there was no significant difference between collagen monofibril diameters between mutant and normal cell lines. Figure 4.5 demonstrates the transmission electron microscopy images used for analysis.

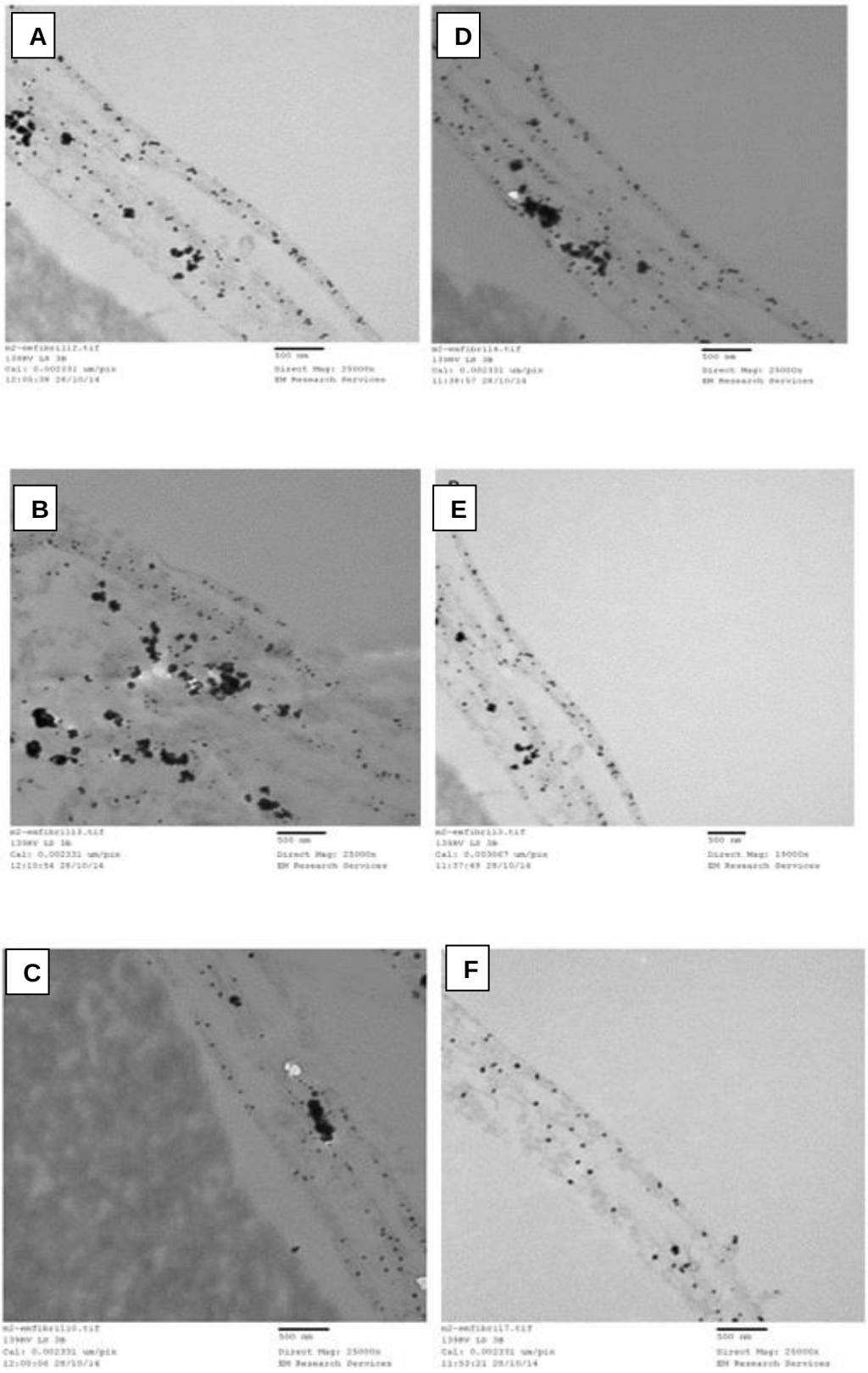
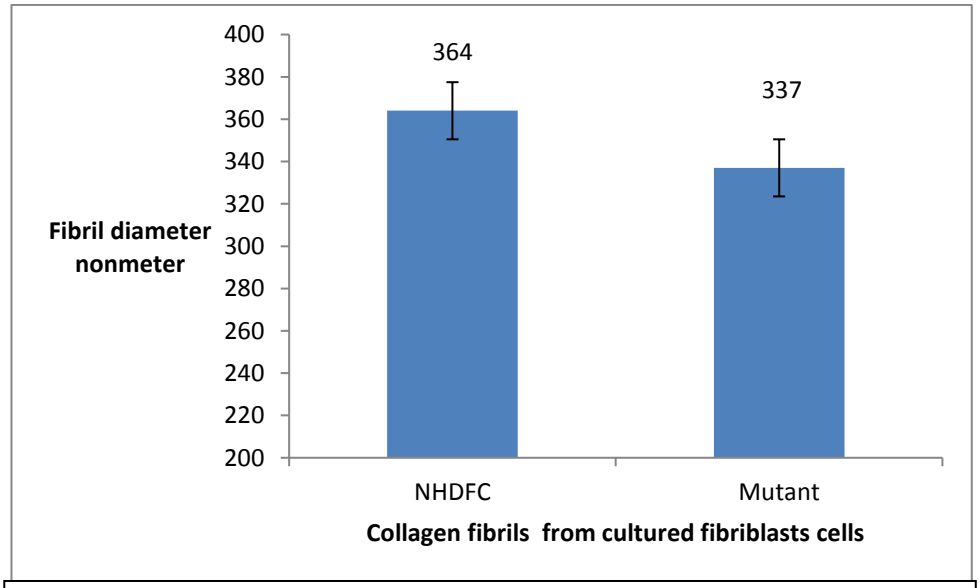


Figure 4.5.Immunogold collagen fibril images. In vivo cultured collagen fibrils from normal human dermal fibroblast cells (*NHDFCs*) is shown as long cylindrical structure. Collagen XII molecule represent on them as black dots (A, B and C). In vivo cultured collagen I fibrils from splice donor mutation is shown as long cylindrical structure (*BM1`)*. Collagen XII molecule represent on them as black dots. Black islets are integration of immunogold particles (D, E and F).

Table 4.1 and graph 4.1 shows the statistical value and comparison in diameter between mutant (BM1`) and control cell lines.

	Experiment 1		Experiment 2		Experiment 3		Total	
	NHDFC	Mutant	NHDFC	Mutant(BM1`)	NHDFC	Mutant	NHDFC	Mutant
MEAN	438nm	373nm	288nm	300nm	362nm	328nm	364nm	337nm
SD	105	123	56.2	32.3	73.5	47.5	84.5	71.3
Min	246nm	211nm	188nm	246nm	278nm	293nm	188nm	211nm
Max	562nm	434nm	387nm	340nm	414nm	361nm	562nm	434nm

Table 4.1 demonstrates the result of fibril diameter measurement of cultured fibroblasts from three different experiments.



Graph 4.1.Statistical analysis of collagen fibrils diameter from cultured fibroblasts. The graphs demonstrated that there is no difference between mutant cells (BM1`) and NHDFCs.

The mean diameter of samples for controls was 364 nm while the average diameter for mutant samples was 337 nm. The experiment of *in vitro* fibrillogenesis was repeated three times. Unpaired t-test reveals there is no siginificant difference

between mutant and normal samples' fibril diameter (P> 0.05). Collagen XII molecules were seen on collagen I fibrils in both mutant and healthy donors. The presence of collagen XII molecules on missplicing donor samples (BM1`) indicates that collagen XII molecule is secreted to ECM in both mutant and healthy donors. However, as electron microscopy is not a quantitative method we could not detect and compare the difference in the amount of collagen XII protein which is secreted into ECM in normal and mutant cell lines in this experiment. Immunoblotting studies for collagen XII protein in BM1`(results showed on chapter 3) represented a reduction on collagen XII protein secreted to ECM. The difference between these two results just echoed the different variations that they measured.

Figure 4.6 shows an image of two fibroblast cells attached to the plastic culture dish. Collagen XII molecules which are under assembly are represented inside cells as black dots. This figure represent the presence and localisation of collagen I fibrils which are neighbouring fibroblast cells and is a confirmation of true detection of collagen I fibrils.

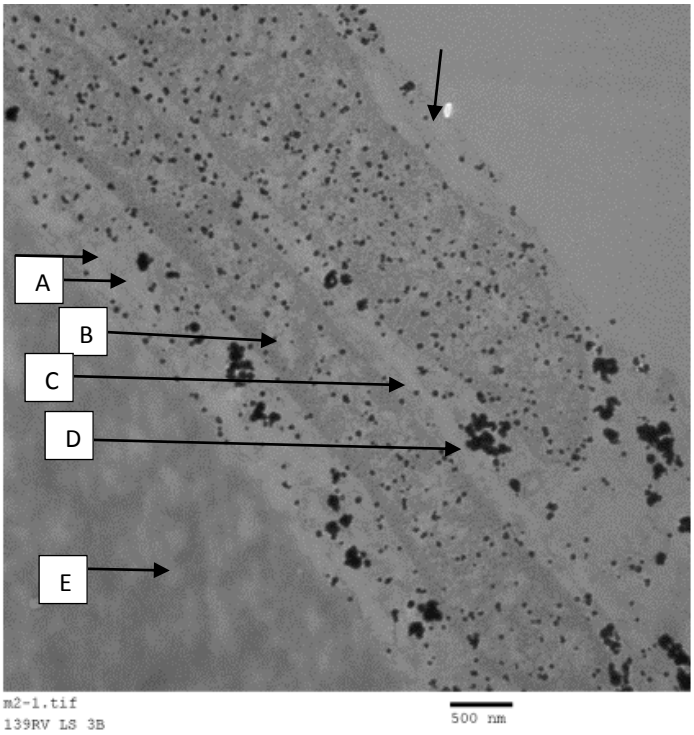


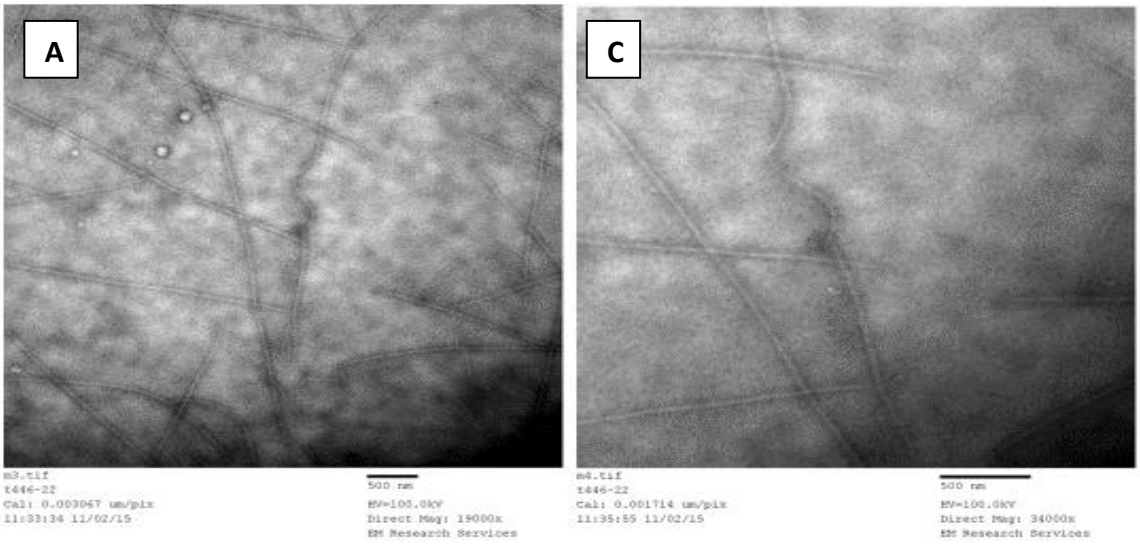
Figure 4.6.Fibroblast cell from the *in vivo* experiment. Vertical arrow represents collagen I fibrils. Horizontal arrows represent cytoplasm of a fibroblast (A), cell nucleus (B), cell wall (C), intercellular space (D), Plastic part of the cell culture well (E). Intracellular collagen XII molecules represent in black dots.

4.3.3 *Electron microscopy evaluation of collagen fibrils in ECM from BM1':*

Following the result of ECM collagen fibril measurements, we embarked on *in vitro* collagen fibrillogenesis investigations. Fibroblasts from patients with a splice donor mutation (BM1') and controls were taken and grown in order to extract collagen from those according to the previously described method (Vomund *et al.*, 2004). This method also described in details in material and method section.

Fibrillogenesis was induced in these collagen solutions by adding 0.5 normal acetic acid under dialysis. Then solutions with collagen fibrils were taken to the Electron Microscopy Unit for negative staining which was described in material and methods section. In this method micro particles were covered with uranyl acetate and one drop of samples were put on grid. These grids then set on electron microscope imaging.

Fibrils were identified as long tubular lines in the images. Figure 4.7, table 4.2 and graph 4.2 shows the resulted fibrils and fibril diameters measurements from *in vitro* fibrillogenesis experiment for mutant and normal cell lines.



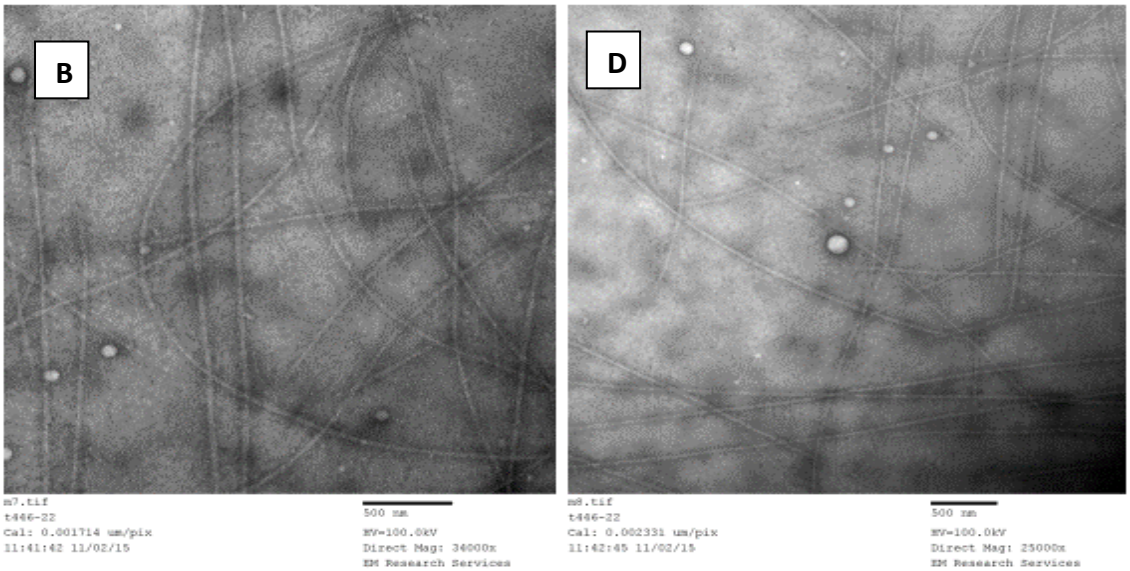
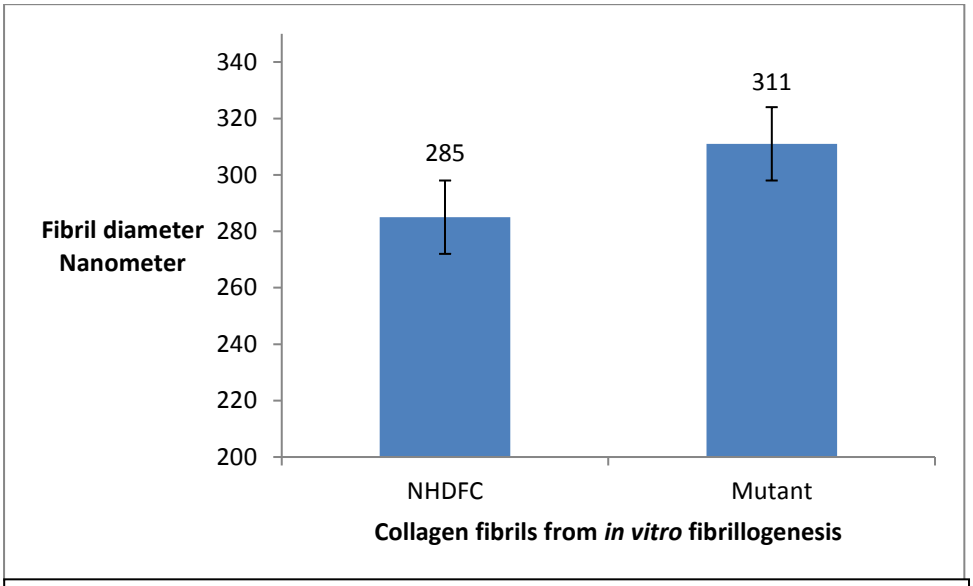


Figure 4.7.Images of induced fibrils. *In vitro* induced fibrils from splice donor mutant sample is shown **(**A, B). *In vitro* fibrils induced from NHDFCs is shown (C, D).Collagen fibrils are presented as long tubular structure.

	Experiment 1		Experiment 2		Experiment 3		total	
	NHDFC	MUTANT	NHDFC	MUTANT	NHDFC	MUTANT	NHDFC	MUTANT
MEAN	229nm	258nm	338nm	296nm	288nm	379nm	285nm	311nm
SD	31.5	48.93	115.7	33.5	54	130.042	97.4	87.9
Min	172nm	188nm	179nm	242nm	186nm	211nm	172	188
Max	352nm	404nm	586nm	342nm	387nm	502nm	586	562

Table 4.2 demonstrates the result of collagen fibril measurements.



Graph 4.2.Comparison of mean fibril diameter between mutant and normal fibrils. The average diameter of mutant and normal control is shown in induced fibrillogenesis experiment.

Also, extracted collagen I (Thermo Fisher Scientific) from a rat tail was used at 1 mg/ml concentration and fibrillogenesis was induced in this sample along with collagen extracted from fibroblasts. The average diameter of 325 was recorded for collagen fibril from rat tail. This commercial sample was used as a positive control for the three independent experiment. Figure 4.8 shows the induced fibrils from extracted collagen I rat tails.

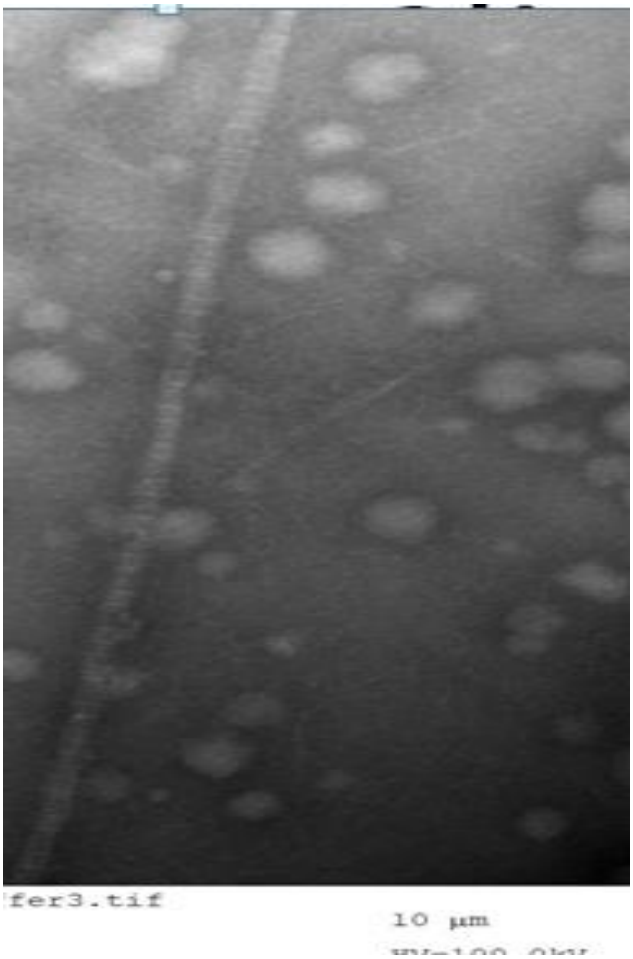


Figure 4.8. An induced collagen fibril from commercial rat tail extracted sample.

The mean diameter of samples for controls was 285 nm while the average diameter for mutant samples was 311 nm. The experiment of *in vitro* fibrillogenesis was repeated three times and each time at least five images from each sample were taken. In each sample two fibrils, which are continued in the whole field, were measured. The horizontal diameter of each fibril was measured with using Image J. The horizontal diameter counted as the shortest distance between two opposite points on the fibril

diameter. The diameters was measured for at least ten opposite points on each fibril started from the part on the highest point of the image to the lowest point. The highest and lowest point have 500 nm distance from image frame line. The distance between highest and lowest point was devided into at least ten part to set opposite points. The examiner tried to keep equal distances. However the distances were not measured with Image J. The diameter between two points were calculated by Image J analyzer. Unpaired t-tests were used to compare diameter in mutant cell lines and normal controls. There was no significant difference between mutant and normal fibril diameters ($P > 0.05$).

4.4 Discussion:

There is evidence that an absence of collagen XII from the environment could lead to thicker collagen I fibrils (Koch *et al.*, 1995). Koch *et al*, extracted collagen I molecules from chick tails. Then induced fibrilogenesis in collagen I molecules in an *in vitro* system. By adding high salt concentration (NaCl) they eliminate collagen XII molecules from the fibrilogenesis environment while a low salt concentration induced collagen I fibrilogenesis in presence of collagen XII. Immunogold antibodies against collagen XII were used to detect presence or absence of collagen XII on *In vitro* synthetic collagen I fibrils. The image of collagen fibrils were taken with Transmission electron microscopy. The images represented thicker collagen I diameter in the absence of collagen XII molecules. We tested this hypothesis in our work, whether structural abnormalities in the collagen XII molecule could lead to thicker collagen fibrils as it is the case for absence of collagen XII. We used samples of splice-donor collagen XII mutation (BM1') along with a control samples (NHDFCs) to do the *in vivo* and *in vitro* experiments. We could not find any differences in collagen fibril diameters between normal and mutant samples.

In order to determine the effect of a *COL12A1* mutation on the ECM we used skin biopsy samples because this is one of the organs which presents with collagen abnormalities. The effect of collagen abnormalities in skin has been described

before. TEM staining for nodules of extracellular matrix showed an altered collagen fibres ultrastructure. Moinzade et al reported that immunohistochemistry staining of the dermis in a scleroderma patient showed collagen XII was deposited in nodules and in the papillary dermis with aberrant forms. Fibrils of thinner and irregular collagen I has a deposition of collagen XII on them (Moinzadeh *et al.*, 2013). In our experiment, we identify that the skin biopsy of *COL12A1* mutant individuals has irregular collagen fibres with breaking in length and irregular cross sectional pattern (Figure 4.1 and 4.2). A basement membrane thickening of the endothelial wall of capillary was also seen. These findings may support the ultrastructural abnormality in keloid scars of patients. However, because we compare scars from *COL12A1* affected individuals with normal part of healthy donors, it might be that the similar changes that we found are variations of every pathologic nodule compared to controls. It would be valuable if we could compare one sample of the skin biopsy of a mutant patient's normal skin region with the same skin location in normal controls. The unavailability of skin biopsy from a normal part of skin in any of our mutant individuals is on limitation in this study. However, this experiment for proving the abnormal collagen fibres characteristics in patients with collagen XII mutation was important. The same features were seen in skin tags of a Bethlem-like myopathy patient with the collagen VI mutation (Kirschner *et al.*, 2005; Starborg *et al.*, 2008a). In consequence, our study of skin biopsy scars` imaging in collagen XII mutant patients represent the same scar abnormalities (variation in collagen fibres diameter, breaks in collagen fibres length and thickening of capillary cell walls membrane) which had been reported in collagen VI patients before.

One other limitation was there was not enough tissue in our samples to do immunohistochemistry staining for collagen XII in skin biopsies. However, Moinzadeh et al *(Moinzadeh et al., 2013)* compared a nodular part of a systemic sclerosis patient with a normal part of a control individual and found different pattern for collagen XII distribution.

After proving the abnormality in the nodular part of the skin in patients with collagen XII mutations, we aimed to identify whether collagen XII plays any role in fibrillary collagen assembly and structural formation. As mentioned earlier, ECM is composed of collagenous and non-collagenous parts. Fibrillar collagens are collagen I, II, III.

These are the most abundant forms of collagen in the ECM. Their assembly in collagen fibrils start from inside the cell and continue in the ECM environment. Collagen fibrils in skin and musculoskeletal connective tissues are mostly composed of collagen I (Halper and Kjaer, 2014).

As mentioned earlier, during fibrillogenesis, long fibrils first bind to each other by non-covalent attachments and then are fixed by covalent attachments. The fibrillogenesis process is a sensitive one and variables such as temperature, other molecules, and ionic exchange could alter that in *in vivo* and *in vitro* conditions(Harris and Reiber, 2007). Formation of alternative association products such as segment long spacing (SLS), fibrous long spacing (FLS), and symmetrical and spiral-banded fibrils in addition to the usual cylindrical fibrils has been reported in experiments that induce *in vitro* fibrillogenesis (Harris *et al.*, 2005). We considered the influence of the environment in fibrillogenesis and to avoid this problem we checked the fibril diameter both *in vitro* and in condition more similar to *in vivo.*

Several studies have reported before that changing the pH or temperature during *in vitro* fibrillogenesis could affect the fibril diameter (Harris *et al.*, 2005; Starborg *et al.*, 2008b). Harris et al checked the effect of pH in extracted collagen from chicken fibrillogenesis. It was found that a pH range of 3.5-8 was capable of fibril formation. However, a lower pH could produce thin and intermediate fibrils while a higher pH produces thicker fibrils (Harris and Reiber, 2007). Collagen fibrils normally have around 250-500 nm diameter (Harris *et al.*, 2005). Different studies suggest that in the physiological pH (7.3) collagen fibrils are around 300-350nm (Harris and Reiber, 2007; Starborg *et al.*, 2008b). In our study, a physiological pH (7.5) was used to produce fibrils at room temperature. The fibrils average of 285 nm (control) and 311 nm (mutant) diameter was found. These findings echoed the average range of fibril diameter in natural conditions. It should be noted that other fibrillogenesis studies did not extract collagen from cultured fibroblasts and mostly used animal sources (Harris and Reiber, 2007). We have a commercial positive control from rat tail extraction which gave a 325 nm diameter under the same experimental conditions as our cell

culture samples. There was no variation in fibril diameter between different repeats of one experiment which shows validity of our experiment. Fibrils have similar structure in all images and no other structures rather than cylindrical fibrils was observed. Also, by inducing fibrillogenesis both *in vitro* and from cultured fibroblasts we tried to avoid temperature and pH side effects. In addition, the fibril diameters for cultured fibroblast experiment were 374 nm (control) and 337 nm (mutant). These values are in normal range and closer to natural condition. This could echo the more similar condition to *in vivo* experiment from cultured fibroblasts.

We used TEM for fibrillogenesis studies. TEM is more suitable for fibrillar supramolecular studies compared to other models such as the X-ray diffraction technique. In the X-ray diffraction technique usually dismiss some features of the ultrastructural model because they are not compatible with the X-ray diffraction technique. These features are normally missed in the X-ray as noise (Starborg *et al.*, 2008b). However, TEM techniques have problems such as artefacts which are caused by dehydration, fixation and sectioning. One substitute technique to avoid this problem is atomic force microscopy in fluid which is not available in our centre (Birk *et al.*, 1997).

We observed one fibril has got variation in its diameter in different points. This is consistent with observations of other experiments which show a discrete rather than continuous distribution for the collagen fibril diameter. This pattern echoed the radial structure of fibrillogenesis where helical layers are laid down concentrically like the growth rings of a tree. These structures are called C-type fibrils and are common in long and heterogeneous collagen I fibrils (Harris and Reiber, 2007). Another factor which might affect collagen I fibril diameter is gold labelling. These2 to 5nm particles could disturb the helical structure due to their anisotropic ability. Moreover, there are amino acid specific binding sites for this particle but there is no evidence that gold particles which label collagen XII could interact with collagen I fibrils *in vivo*. There was no, uranyl acetate which is used to stain collagen I fibrils *in vitro* could permeate into the fibril or on the surface of fibrils it may interact with periodically arranged amino acid chains. In this aspect, our cultured fibroblasts experiment has the value

to reduce these side effects. Koch et al reported that in the absence of collagen XII, collagen I fibrils have larger diameters (Koch *et al.*, 1995). In that experiment TEM with post-embedding immunogold staining was used to image collagen I fibrils in the absence and presence of collagen XII. The reason for this observation remains to be discovered. In our experiment, we test whether collagen fibrils in the extracellular matrix of the patient with a splice donor mutation (BM1') are thicker compared to collagen fibrils in the *in vitro* experiment. Immunoblotting of this sample also showed a reduction in the amount of collagen XII protein secreted to the media (medium protein extraction) while the total amount of protein (cell culture extraction) is significantly increased. This pattern as previously discussed suggests that despite the mutation does not affect the production of collagen XII; the product which is secreted to ECM is unstable and could not assemble into ECM structure completely. We also described collagen XII immunofluorescence staining pattern for BM1` as highly reduced collagen XII signals both intra and extracellular, because not only fibrillar diffuse pattern of collagen XII is missed but also the available signals is limited only around the nucleus which is incompetent and insufficient for intracellular retention. Our immunogold experiment showed collagen XII has localized on collagen I fibrils in both mutant samples and control. This pattern is not compatible with immunofluorescence staining. The reason for that is immunogold antibody for electron microscopy has higher affinity that even tiny amount of proteins would be picked but in order to have signals of that protein in immunofluorescence staining very larger amount of protein with correct structure is required. However, it is impossible to quantify the amount of collagen XII between the two samples in this electron microscopy study. Generally, electron microscopy is suitable for investigating the pattern and morphology of different structures –not for quantification. The lack of ability to quantify the total amount of protein is a limitation in our *in vivo* imaging of collagen fibrils by electron microscopy. Our fibrillogenesis investigation does not show any difference in the presence of the collagen XII molecule on collagen I fibrils in *in vivo* ECM samples. Koch et al had used an *in vitro* method to extract collagen and in order to prevent potential changes due to its preparation for electron microscopy they used the post-embedding method. The situation is of the absence of collagen XII compared to its presence in that work

(Koch *et al.*, 1995). We want to check whether mutant samples could affect fibril diameter by not attaching to them.

Our results suggests that even if our mutant collagen XII molecule could affect collagen fibril characteristics, this effect does not change the diameter. There is no discrepancy between cultured fibroblast and *in vitro* fibrillogenesis experiments.

One limitation in our study is that due to a lack of facilities and time we could not evaluate other aspects of collagen fibrils such as collagen fibrils length *in vivo* and *in vitro*. It has been reported by Zuo et al that collagen XII-deficient mouse muscle had reduced strength force measurements (Bolduc *et al.*, 2014; Zou *et al.*, 2014). Tissue stiffness is controlled by lateral fibril connections. As previously described 3Dimension (3D) electron microscopy reconstructions were able to demonstrate lateral connections of fibrils which are called banded fibrils. Even a small change in this lateral connection could make samples stiffer. Collagen XII as a FACIT collagen, which facilitates the interaction between collagen fibrils and other ECM components, may play a role in lateral banding (Starborg *et al.*, 2008b). 3D construction with electron microscopy in our fibroblast cultured experiment may show some differences in lateral banding pattern However, the complicated procedure of preparation and analysis of images with the 3D method was beyond our laboratory's potential.

The other aspect which could be evaluated is the longitudinal length of fibrils. The total length of fibrils varied among different tissues and it shows that in more elastic tissues such as the tendon a total length of a fibre could be as long as the tendon itself. There are limited studies about the effect of fibril length in tissue characteristics. However, there is a possibility that a reduction in tissue elasticity may occur by a change in total fibril length. Recently discovered methods such as continuous trans-sectional electron microscopy were used to identify collagen fibril

length. However, these methods need specific instrumentation and techniques. Besides that, they are time-consuming and expensive.

One other limitation is that skin biopsies from all *COL12A1* affected individuals were not available for imaging. It worth to check variations and similarities of ultrastructural skin involvements in these patients. In addition due to financial and time limitation we could not include all mutant samples in our fibril dimeter measurements. Splice donor mutation was a random selection.

We evaluated the effect of collagen XII mutation in the collagen I fibril diameter in vitro and in cultured fibroblasts by electron microscopy. This study did not identify any differences between the two samples but other aspects of collagen fibrils in tissues with the collagen XII mutation remain to be evaluated.

Chapter 5 *COL12A1* mutant fibroblasts effect on myofibres differentiation

5.1 Introduction: Muscle growth and repair mechanisms

Ullrich congenital muscular dystrophy (UCMD) and Bethlem myopathy (BM) are two types of collagen VI-related myopathies that demonstrate the role of collagen VI as an important extracellular matrix (ECM) component in muscle disorders (Bonnemann *et al.*, 2011). However, the exact pathomechanisms of these muscle diseases remains unknown (Bonnemann, 2011). Given the fact that Collagen VI as a disease-causing gene for neuromuscular disorder is probably secreted from interstitial fibroblasts of ECM, the issue of myocyte/ECM interaction in muscle disorders becomes more important. However, both biomechanical and chemical properties of this interaction between myocytes and extracellular matrix remain yet to be discovered(Lampe *et al.*, 1993). Myocyte/ECM interaction in tissues which are tolerating extensive mechanical stress, such as skeletal muscle is more important (Sabatelli *et al.*, 2012b). In order to understand the mechanism of this interaction, we need to introduce the process of muscle development and regeneration.

Myocytes are the primary cell source for developing muscle tissue. When myocytes devide and align to each other in a fashioned way, they make myotubes. Myotube development leads to muscle fibre creation (Urciuolo *et al.*, 2013). Differentiation of myotubes in an *in vitro* model starts when myocytes become angular and attach to their neighbouring cells. Myotubes are considered as the functional unit of muscle tissue and are derived from fusion and differentiation of myocytes(Piccolo *et al.*, 1995). Figure 5.1 represents a picture of myocyte differentiation.

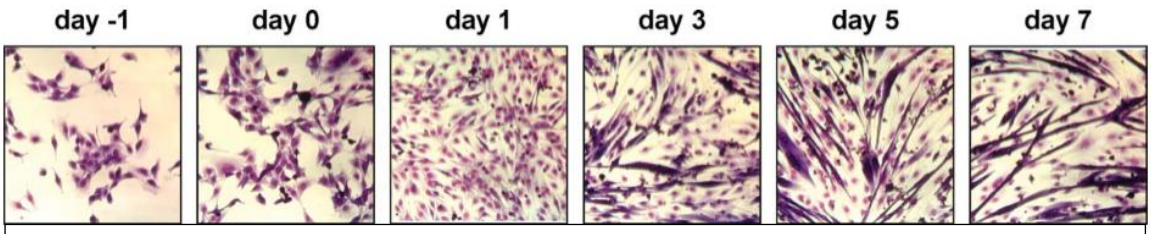


Figure 5.1. C2C12 differentiation stages according to number of days cells stayed in culture.

Quiescent satellite cells are the precursor of myocytes and play an additional role in ECM. During muscle injuries, these quiescent cells induce proliferation and migration of myocytes to the site of muscle injury. Regarding this, myocytes are considered as the main source of muscle regeneration and repair during injury (Rao *et al.*, 2013a; Rao *et al.*, 2013b). Quiescent satellite cells have a large capacity to induce myocyte migration to the site of injury. Occasionally myocytes are unable to fully repair the damage and partial repair happens in some areas. In these regions the fibrotic scars, which derive from connective tissue, substitute for muscle fibres. Thus, fibrosis is not only a sign of incomplete muscle repair but also a sign of a muscle disorder(Rao *et al.*, 2013a). Fibrosis could happen both in ECM related muscle disorders as well as non-ECM related muscle weaknesses (Sabatelli *et al.*, 2012b).

Recent studies suggest that muscle repair is a complicated process which involves different components apart from myocytes and satellite cells. Fibroblasts also play an important role in muscle regeneration and repair (Urciuolo *et al.*, 2013). Role of fibroblast which localized in ECM in regeneration and repair could be due to their signalling role and secretion of different molecules (Sabatelli *et al.*, 2012b). One hypothesis is that these molecules could help to provide quiescent satellite cells with a stable environment during regeneration or induce myocytes activities (Urciuolo *et al.*, 2013). Another potential way for fibroblasts to interact in muscle regeneration is through direct contact. Interstitial fibroblasts are neighbouring satellite cells and from their apical surface are in contact with the basement membrane (Urciuolo *et al.*, 2013). These studies shed light on the important role of fibroblast ECM in muscle regeneration, repair, and muscle disorder pathogenesis.

The potential relationship between fibroblasts and muscle tissue has been investigated in different studies (Rao *et al.*, 2013a; Rao *et al.*, 2013b; Urciuolo *et al.*, 2013) .In the presence of fibroblasts *in vitro*, cardiomyocytes changed morphologically and in contractile ability (Mann *et al.*, 2011). Another study showed that coculturing cardiac fibroblasts with cardiomyocytes reduces misalignment and apoptosis rate in cardiomyocytes (LaFramboise *et al.*, 2007). Furthermore, implantation of fibroblasts with cardiomyocytes during heart injury repair increases the scar repair rate (Nichol *et al.*, 2008).

While these studies provide evidence of positive role of fibroblast on myocyte growth and regeneration during coculture experiments, on the contrary, for disease models such as arrhythmia, coculturing fibroblasts with myoblasts can have a negative effect on repair. Fibroblasts tend to secrete some extracellular matrix proteins and these proteins first inhibit normal apoptosis in different cells which lead to an induction of scar. The second effect of these fibroblasts is to increase the distance between cardiomyocytes which interferes with their normal growth rate (Mann *et al.*, 2011). There is a possibility that fibroblasts in muscular tissue might affect muscle growth similarly. Use of fibroblasts in muscle progenitor cell growth opens new areas in the muscle repair and reproduction field. Myoblasts and satellite cell have been used in skeletal muscle repair research. These researches showed some positive effects of muscle progenitor cells in repair. However, the lack of enough delivery, high apoptosis rate and low quality is visible (Nichol *et al.*, 2008).

In addition to coculture models for investigating myocyte/ ECM interaction during muscle growth and regeneration, the independent effect of fibroblasts on myoblast growth and differentiation has been studied. Independent studies of myoblasts growth and differentiation widely use C2C12 cells (Ricotti *et al.*, 2013; Muthuraman, 2014b). As a cell line, C2C12 was introduced in 1985 by Blau et al from the murine fibroblast cell line. This cell line is a subclone of satellite cells which are considered as precursors of myogenic growth and repair. Because of rapid growth and differentiation ability of C2C12, these cells are commonly used in muscle growth and repair studies (Velica and Bunce, 2011). One study showed that C2C12 cells which grow on top of primary fibroblasts have a lower apoptosis rate. The alignment in C2C12 myotubes also increased (Muthuraman, 2014a).A recent new study checked the fibroblast/myoblast coculture with a reconfigurable system device which could help to check whether the effect of one cell line in another is due to paracrine secretion or direct contact, also it helps to isolate the cell line of interest and check the growth or differentiation of one cell line in the presence of the other (Ricotti *et al.*, 2013).

Another study has demonstrated that fibroblasts could inhibit primary myoblasts growth independent of contact. However, the presence of fibroblasts in close proximity to myoblasts could regulate differentiation and alignment in myoblasts.

These effects depend on the presence of both cell lines in the culture dish. The use of conditioned media from one into another culture could not show the same effect. Also, they demonstrate that transforming growth factor β (TGTβ) which is secreted from fibroblasts plays an important role in primary myoblasts growth. The effect is not independent of contact between fibroblasts and myoblasts (Melone *et al.*, 2000).

The effect of fibroblasts on myoblast growth and regeneration has been studied in Duchene Muscular Dystrophy (DMD) patients. Dystrophin which was identified in 1986 provided an insight into the pathomechanism of DMD in human and animals (Melone *et al.*, 2000). Dystrophin, an important component of plasma membrane, is crucial for preventing muscle necrosis. Recently published studied demonstrated that primary DMD fibroblasts/ myoblast coculture had a higher growth rate *in vitro* compared to primary DMD myoblasts alone (Melone *et al.*, 2000). Moreover, TGF-β1 in excessive amounts induced defective DMD myoblasts production. Moreover, IGF-binding proteins (IGFBP) are of considerable interest because they could bind with high affinity to IGF growth factors such as IGF-I and IGF-II. These complexes thus could change the capacity of cell membrane to bind to other proteins and change its growth action. Some studies suggest that IGFBP-3 could inhibit or induce IGF-I DNA synthesis depending on experimental conditions (Melone *et al.*, 2000; Muthuraman *et al.*, 2014).Several studies model *in vivo* ECM/ myocytes interaction through primary fibroblast/ primary myoblast coculture or adult fibroblasts/primary myoblast coculture .However, the effect of adult fibroblasts in C2C12 growth and differentiation has not been investigated yet (Zou *et al.*, 2008; Rao *et al.*, 2013a).

In these interactive coculture systems collagen molecules are important key factors. Regarding the role of collagen VI, a wealth of observation from different studies suggests that this molecule is an important member of the satellite cell niche for producing ECM components (Zou *et al.*, 2008; Bonnemann, 2011). Like other collagen molecules, collagen VI is secreted by interstitial fibroblasts in satellite cell niche and its expression might be regulated by myogenic cells. These cells are also crucial for deposition of collagen VI on cell membrane (Urciuolo *et al.*, 2013). The anatomical region of the satellite cell niche is between the plasma membrane and endomysial tissue of the muscle fibre. Asymmetrical distribution of muscle cells and their contact with apical part of satellite cells is the anatomical characterization of

satellite cell niche (Melone *et al.*, 2000). Also, in the satellite cell niche muscle elements are in contact with ECM from their basal part. Transcription factors like Paired box protein 7(PAX7), MyoG and MyoD control growth and differentiation of satellite cells. The role of these factors in collagen VI related disease has been investigated, recently (Urciuolo *et al.*, 2013). In the muscle regeneration process, satellite cells play a key role by producing two different cell lines. Highly differentiated primary cell type which derives to myoblasts and a population of stem cells. The myoblast later under goes differentiation and fusion process, while the stem cell population under goes self-renewal. Satellite cells growth, differentiation and activities are controlled by the ECM components. One of the key factors in this regulatory process are the mechanical properties of ECM. Regarding this factor, the role of collagen VI in changing ECM mechanical stiffness could also influence satellite cell activities (Sabatelli *et al.*, 2012b; Urciuolo *et al.*, 2013).

Recent studies suggest that collagen VI is an essential part of the satellite niche in ECM which helps to preserve satellite cell activities such as muscle regeneration and self-renewal (Urciuolo *et al.*, 2013). Urciuolo et al showed in *COL6A* $^{-/-}$ mice grafting with wild type fibroblasts which result in collagen VI deposition could rescue satellite cell self-renewal and muscle stiffness (Urciuolo *et al.*, 2013).

These findings confirm previous findings of Zou et al (Zou *et al.*, 2008) which indicate interstitial fibroblast as the primary source for secretion of collagen VI in ECM. In their study, they cultured human derived fibroblasts with primary mouse myogenic cells. The two cells were cultured in inserted cell culture wells, under two different conditions that two cell culture mediums were mixed or kept independently. They found in both conditions secreted collagen VI was able to deposit on myocytes. A very little amount of collagen VI synthesis was also reported in C2C12 which reflected the dedifferentiation state in these cells (Zou *et al.*, 2008).

We recently demonstrated collagen XII as disease-causing gene in six patients with Bethlem-like myopathy symptoms (muscle weakness, skin and skeletal abnormality, joint hyperlaxity) from three different pedigrees (Hicks *et al.*, 2014). Collagen XII as part of extracellular matrix component shows cell membrane localization in zebrafish model and our mouse muscle staining which resembles collagen VI localization

(Bader *et al.*, 2009; Telfer *et al.*, 2010). Telfer et al, showed *COL6A1* expressed in skin basement membrane near the dermal-epidermal junction and colocalized with Laminin (Telfer *et al.*, 2010). Bader et al, reported the presence of *COL12A1*short isoform in zebrafish model which started expression from emberynic time. Collagen XII was expressed in skin in basement membrane of dermal-epidermal junction, in other connective tissues such as skeletal muscle it express in connective tissue surrounding muscle fibers. In addition collagen XII was localized on collagen fibrils. It is colocalized with Laminin which is a basement membrane marker but its expression is more than Laminin (Bader *et al.*, 2009)Given the fact that all these patients have symptoms of ECM defect and collagen XII is also an ECM component, the question of whether collagen XII induces a muscle disorder with the same pathogenesis becomes an important issue (Chiquet *et al.*, 2014).

Many *in vitro* studies of ECM/ myocyte interaction have used C2C12 myoblast cell lines. In these cells myogenic differentiation and fusion between myoblasts to form multinucleated myotube is induced after changing high-serum culture medium (10% foetal bovine serum) to low-serum culture medium (2-5% horse serum). During the differentiation process, myotubes elongate and express several proteins such as myosin, α-actin, troponin and other contractile components (Urciuolo *et al.*, 2013). In order to quantify myotube differentiation in *in vitro* studies, the total level of these myotube specific proteins is measured. One other widely used method to quantify myotubes differentiation is a fusion index calculation. In this method, after staining myotubes with antibodies against myotubes specific proteins, the number of nuclei inside myotubes is counted (Velica and Bunce, 2011).

In this study, we investigate the effect of fibroblasts of mutant *COL12A1* individuals on C2C12 cell growth and differentiation and compare it to controls. Fibroblasts were cocultured with C2C12 cells. Two methods of coculture were used. One of them, allows direct contact between two cell lines, whereby, a mixture of two cell types (fibroblasts, myoblasts) in direct contact is used to investigate myocytes/ fibroblast interactions. For example, Melone et al, in an *in vitro* model of Duchenne Muscular Dystrophy (DMD) fibroblasts cocultured with primary myoblasts, represent an increased expression of Insulin Growth Factor-Binding protein 5 (IGFBP5) and downregulation of DMD myoblasts growth were observed.

The second method, used by Zuo et al, used cell culture inserted plates to coculture Normal Human Dermal Fibroblasts cells with mouse primary cell lines. NHDFCs were cultured in inserts while mouse primary cells were cultured in cell culture plates. In this system the two cell culture mediums were mixed. They found collagen VI deposition on myoblasts and identified fibroblasts as the primary source for collagen VI secretion (Zou *et al.*, 2008).

These studies helped us to design our coculture methods. We aim to determine the influence of NHDFCs and mutant *COL12A1* fibroblasts on C2C12 differentiation. This influence has been investigated through two coculture systems. One of the systems investigated myotube differentiation through direct contact and another one help to investigate the effect of two culture mediums on C2C12 growth and differentiation. For determining myotubes differentiation, we stained cells with an anti-actin antibody and calculated the myotubes differentiation index. The two methods and achieved results will be described later in this chapter.

5.2 Aim

In this chapter we aim to:

Calculate C2C12 differentiation index when cocultured with *COL12A1* affected individuals and healthy donors in a mixture (direct contact)

Calculate C2C12 differentiation index when cocultured with *COL12A1* affected individuals and healthy donors in inserted cell culture dish (without direct contact)

5.3 Results:

5.3.1 *Fibroblast/ C2C12 coculture in a mixture with COL12A1 mutant individuals' fibroblast:*

C2C12, one control fibroblast and DMD fibroblasts cell lines were obtained from biobank in the MRC Centre for Neuromuscular Diseases Newcastle. One commercial fibroblast control (NHDFC) was purchased from Promocell. Fibroblast cells of different origin (Biobank control, Commercial control), DMD with deletion in exon 49 and 50, mutant fibroblast of BM5 and mutant fibroblast of BM1`were seeded in slide flasks. Dulbeco`s modified eagle medium (DMEM) composed of 10% foetal bovine serum (FBS) and penicillin/streptomycin was used to grow cells. In muscle

biopsies the interstitial fibroblasts range from 20% to 70% (Melone *et al.*, 2000). In order to model *in vivo* extracellular matrix system in an *in vitro* model, fibroblasts were grown to 50% confluency. At this point cells were treated with mitomycin (Sigma-Aldrich) to prevent mitotic activity. Then C2C12 cells were added to slide flasks on top of the fibroblast layer. Growth media was switched to DMEM with 10% horse serum. 24 hours later high serum medium was replaced with low serum (3% horse serum). Substitution of cell culture medium induces C2C12 differentiation to myotubes.

On the day six of differentiation, cells were fixed with Paraformaldehyde (PFA) and stained with phalloidin and DAPI to determine myotubes actin and cell nuclei. Images were taken with Zeiss immunofluorescence microscope. Five independent images were taken from each slide. The experiments were repeated three times. Figure 5.2showes the results for six cell lines from three different experiments. In all experiments the fibroblast and C2C12 were grown under the condition of direct contact between cell layers.

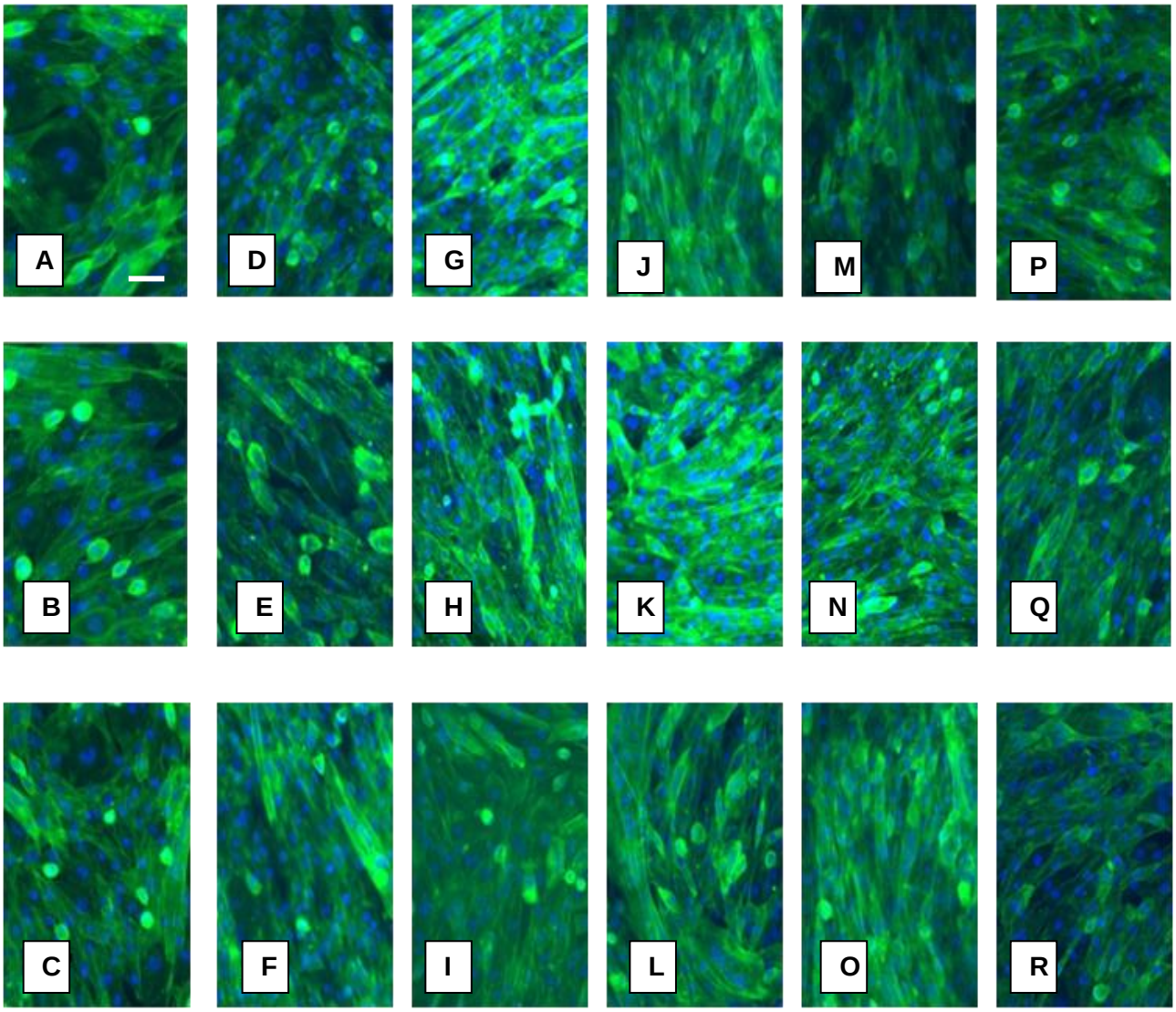
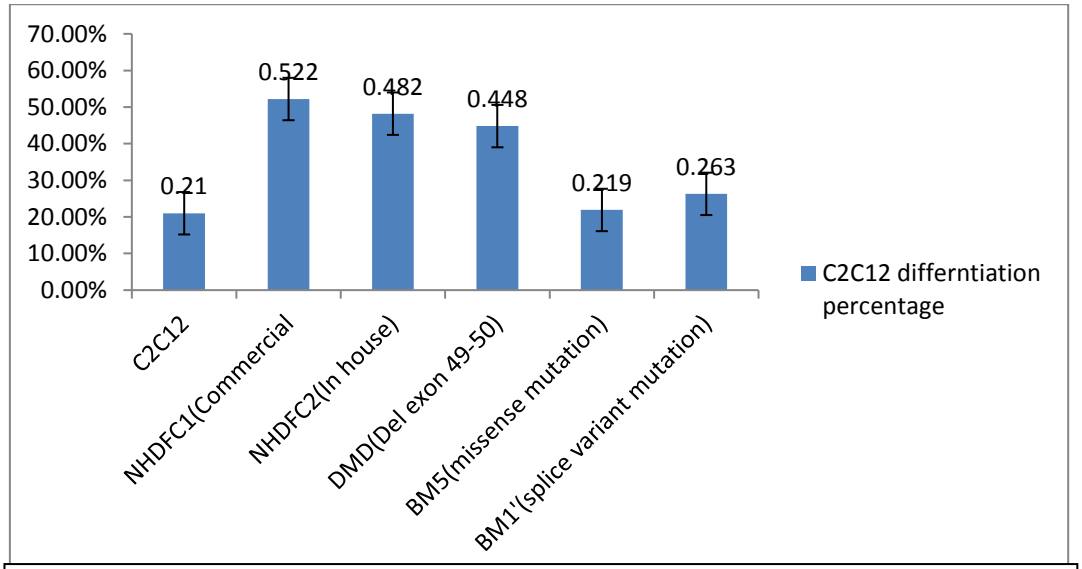


Figure 5.2.Phalloidin staining for Fibroblast/ C2C12 coculture mixture. Actin fibres were imaged in green with phalloidin and cell nuclei were imaged in blue with DAPI. (A, B, C) mutant BM5 fibroblast/ C2C12 coculture. (D, E, F) mutant BM1` fibroblast/ C2C12 coculture. (G, H, I) positive control: DMD fibroblast/ C2C12 coculture. J, K, L) Control fibroblast (commercial)/ C2C12 coculture. (M, N, O) Control fibroblast (Biobank)/ C2C12 coculture. (P, Q, R) Negative control: only C2C12 culture. Images showed three independent experiments (n”3).Scale bar in A = 100 µm.

The differentiation index in each image was calculated to determine the effect of fibroblasts on C2C12 myoblast cell culture. Number of nuclei inside myotubes were counted manually and divided by the total number of nuclei in each image. Table 5.1 showed C2C12 differentiation indexes for three independent experiments, mean and standard deviation for each cell lines.

	C2C12	NHDFC1	NHDFC2	DMD	BM5	BM1`
Experiment 1	0.23	0.546	0.474	0.441	0.228	0.271
Experiment 2	0.215	0.518	0.491	0.453	0.211	0.252
Experiment 3	0.185	0.527	0.480	0.450	0.218	0.266
Mean	0.21	0.522	0.482	0.448	0.219	0.263
SD	0.022	0.014	0.016	0.017	0.014	0.023

Table 5.1. Nucleus differentiation index for six different cell lines in mixture coculture. Graph 5.1 showed total percent of nuclei inside myotubes for six groups of fibroblast/C2C12 coculture: Mutant BM5, mutant BM1`, positive control of DMD fibroblasts, Normal Human Dermal Fibroblast Cell (NHDFC), Biobank control, negative control of C2C12.



Graph 5.1.Myotubes differentiation index in mixture coculture with fibroblasts. Myotubes differentiation index as Percentage of nuclei inside myotubes is shown in vertical graph. Six different cell types: BM5, BM1`, DMD, NHDFC1, NHDFC2, C2C12 alone were compared to each other.

Two proportional t- test was used to calculate P-value of *COL12A1* mutant fibroblasts compare to control cells (NHDFC1+NHDFC2), C2C12 and DMD fibroblasts. In addition, differentiation index of DMD fibroblasts were compared to normal controls (NHDFC1+NHDFC2) and C2C12 cells. P-value for*COL12A1*mutant cells compare to normal control and C2C12 was P< 0.01. While P-value for *COL12A1* mutant fibroblasts compare to DMD fibroblasts was P>0.05. P-value of DMD fibroblasts compare to normal control and C2C12 was P>0.05.

Statistical analysis was done with SPSS software. Statistical analysis shows NHDFC/ C2C12 coculture increase C2C12 differentiation compare to *COL12A1* mutant/ C2C12 coculture or other type of the cells coculture. *COL12A1* mutant/ C2C12 coculture did not influence C2C12 differentiation compare to C2C12 culturing alone. DMD fibroblasts/ C2C12 coculture also did not affect C2C12 differentiation compare to C2C12.

5.3.2 *Fibroblast/ C2C12 coculture without direct contact inCOL12A1 mutant individuals' fibroblasts:*

In the previous experiment, the effect of fibroblast /C2C12 coculture through direct contact between two cell types was investigated. The present experiment evaluated whether coculturing C2C12 with medium sharing fibroblasts influence C2C12 differentiation index. Five previously described control and mutant fibroblasts: BM5, BM1`, DMD, Commercial control, Biobank control, C2C12 were grown on an inserted 12 cell culture dish.

Fibroblasts (passage<5) were grown on top of wells with DMEM growth media composed of 10% foetal bovine serum and penicillin/streptomycin solution. Fibroblasts were grown to 50% confluency and were treated to inhibit mitotic activity with mitomycin (Sigma-Aldrich).12 hours later, C2C12 cells were seeded on the bottom of each well and muscle growth media was added.24 hours after seeding C2C12, myoblast growth media was switched to myoblast differentiation media (3% horse serum) to induce differentiation.

On the sixth day of differentiation, C2C12 cells were fixed using PFA and stained with DAPI and phalloidin in the cell culture dish. Images were taken with Nikon

confocal microscope. Five independent images were taken from each well. The experiments were repeated three times. Images for six cell lines from three independent experiments were shown in figure 5.3.

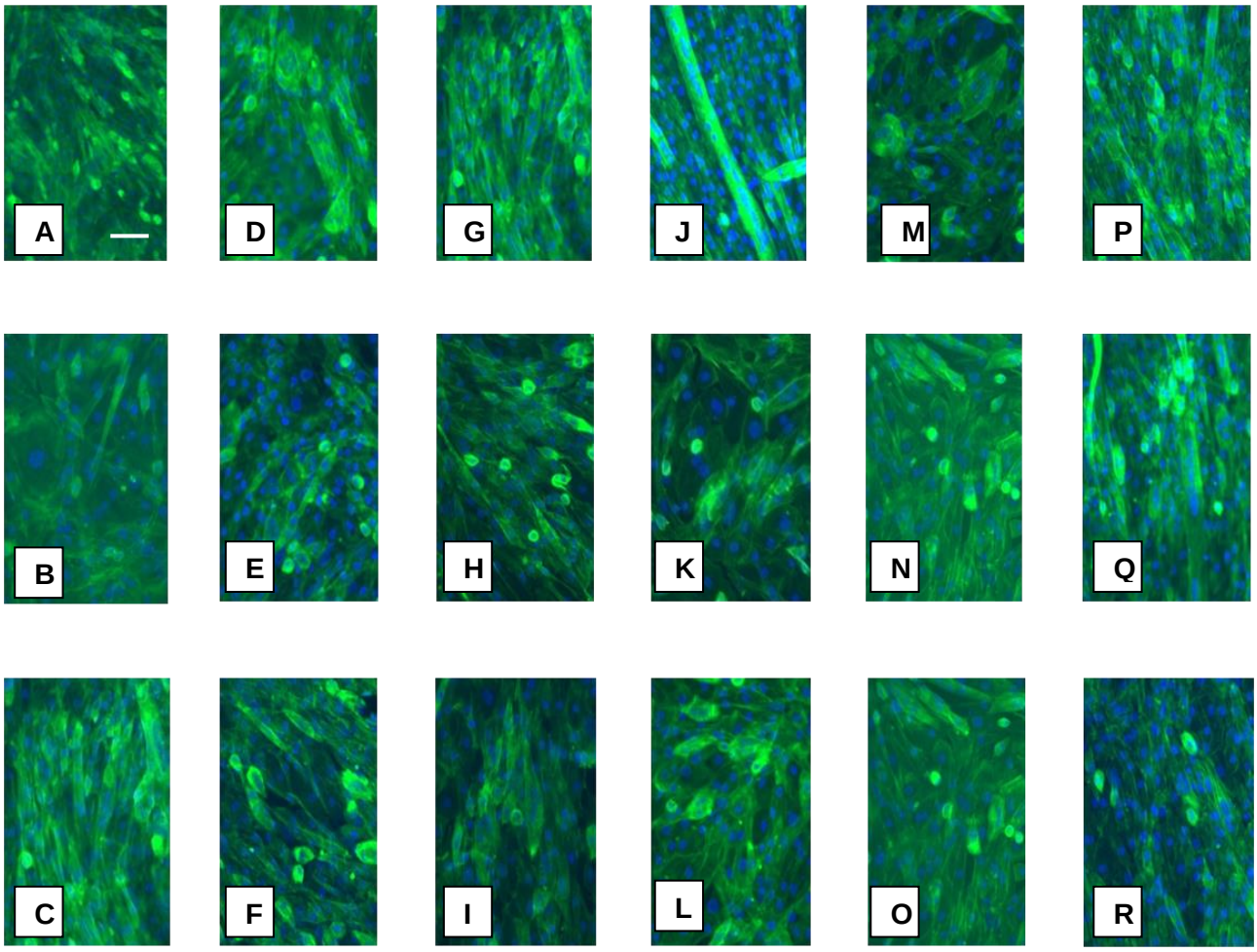


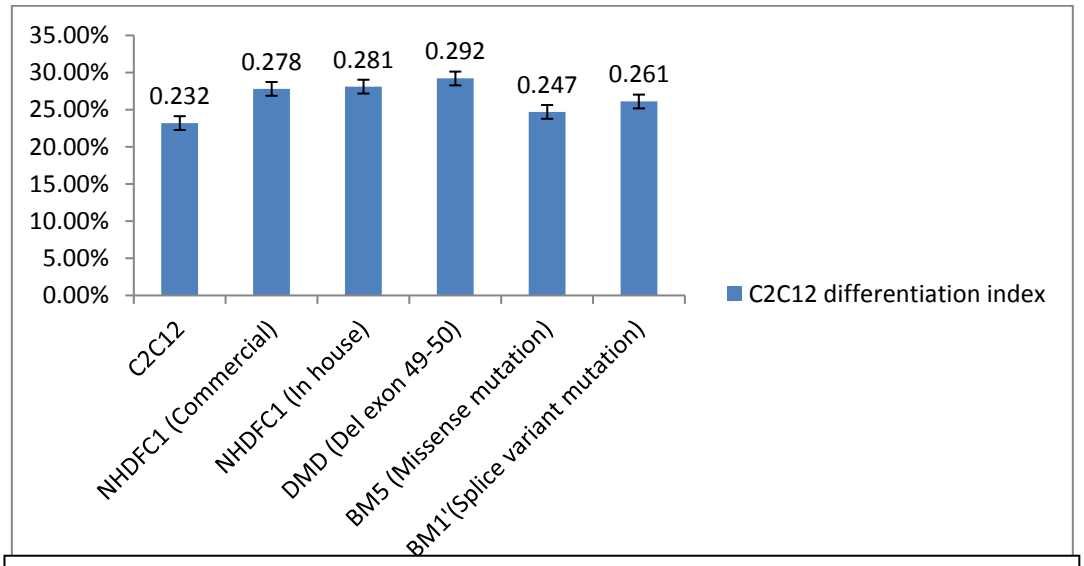
Figure 5.3.Phalloidin staining for Fibroblast/C2C12 coculture in inserted cell culture dishes. Actin fibres were imaged in green with phalloidin and cell nuclei were imaged in blue with DAPI. (A, B, C) mutant BM5 fibroblast/C2C12 coculture. (D, E, F) mutant BM1` fibroblast/ C2C12 coculture. G, H, I) positive control: DMD fibroblast/C2C12 coculture. (J, K, L) Control fibroblast (commercial)/C2C12 coculture. (M, N, O) Control fibroblast (Biobank)/C2C12 coculture. (P, Q, R) Negative control: only C2C12 culture. Images showed from three independent experiments. Scale bar in A = 100 µm.

The differentiation index in each image was calculated manually by counting the number of nuclei inside myotubes and divided by the total number of nuclei in each image. Table 5.2 showed nucleous differentiation index, mean and standard deviation for three independent experiments in six cell lines.

	C2C12	NHDFC1	NHDFC2	DMD	BM5	BM1`
Experiment 1	0.245	0.289	0.288	0.295	0.256	0.271
Experiment 2	0.224	0.281	0.293	0.284	0.234	0.266
Experiment 3	0.233	0.264	0.262	0.297	0.251	0246
Mean	0.232	0.278	0.281	0.292	0.247	0.261
SD	0.010	0.012	0.016	0.007	0.011	0.013

Table 5.2 Nucleus differentiation index for six different cell lines` coculture without direct contact.

Graph 5.2 shows total percent of nuclei inside myotubes for six groups of cocultures: Mutant BM5, mutant BM1`, positive control of DMD fibroblasts, Normal Human Dermal Fibroblast Cell (NHDFC), Biobank control, negative control of C2C12.



Graph 5.2.Myotubes differentiation index in coculture with fibroblasts but no direct contact. Myotubes differentiation index as Percentage of nuclei inside myotubes is shown in vertical graphs. Six different cell types: BM5, BM1`, DMD, NHDFC1, NHDFC2, only C2C12 were compared to each other.

Differentiation index of*COL12A1* mutant fibroblasts was compared to normal controls (NHDFC1+NHDFC2), C2C12 and DMD fibroblasts. In addition, differentiation index of DMD fibroblasts were compared to normal controls (NHDFC1+NHDFC2) and C2C12 cells. Two proportional t-test was used to calculate P-value. P-value for *COL12A1* mutant cells compare to normal controls, DMD fibroblasts and C2C12 was P> 0.05. Also, P-value of DMD fibroblasts compare to normal control and C2C12 was P>0.05.

Statistical analysis shows fibroblast/ C2C12 coculture in an inserted well does not influence C2C12 differentiation index in*COL12A1* mutant fibroblast/ C2C12 coculture or other type of the cells coculture.

5.4 Discussion:

Different studies suggest that in collagen VI-related myopathies, the cells that cause disease (fibroblasts) are different from the cells that express the disease (muscle) (Lampe *et al.*, 1993; Zou *et al.*, 2008). Given this fact, collagen VI-related myopathies and in a broader spectrum ECM disorders are considered to be non-cell autonomous disorder and investigating pathogenesis mechanisms of these disorders via coculture systems has become increasingly important.

In this chapter, we investigated the influence of fibroblasts on C2C12 differentiation. In order to model *in vivo* conditions in an *in vitro* experiment, we cocultured fibroblasts and C2C12 in a mixture of different cell layers with direct contact. This experiment shows coculturing C2C12 with affected individuals' fibroblasts decreases C2C12 differentiation index. The second step, was designed to understand whether physical contact between two cell types induces reduction in C2C12 differentiation or an indirect system through chemical substances or a signalling pathway affected C2C12 differentiation. Second experiments showed coculturing C2C12 and fibroblasts in separated wells while they share same media does not affect C2C12 differentiation index for affected individuals compare to control subjects.

Previous studies in collagen VI-related myopathies suggested that coculturing fibroblasts and mouse primary myogenic cells in inserted wells in different levels and mixture of two cell types in the same dish, similar to our coculture system, could result in interaction between collagen VI secreted from normal human fibroblasts and primary myogenic cells (Zou *et al.*, 2008; Urciuolo *et al.*, 2013). Furthermore, Zou et al (Zou *et al.*, 2008) identified deposition of collagen VI in interstitial fibroblasts and C2C12 independent cell cultures but not in primary myogenic cell cultures. In addition, they recognized retention of collagen VI in interstitial fibroblasts of three mutant collagen VI (*COL6A1, COL6A2, COL6A3*) patients but not in their myofibers. This study implies interstitial fibroblasts as the main source of collagen VI secretion in an *in vitro* model of muscle extra cellular matrix (ECM). These results could show interactions between adult human fibroblasts and primary mouse myogenic cells in the context of collagen VI related myopathies in an *in vitro* model and open a new window for investigating collagen VI- related myopathies and other ECM disorders through coculture systems.

In this experiment, we used adult human fibroblasts and C2C12 for coculture. Use of adult human fibroblast and primary myogenic cells or primary fibroblast and primary myogenic cells in muscle disorder research or other research has been reported (Ciofani *et al.*, 2010; Urciuolo *et al.*, 2013). One concern in using cells from different developmental origin is whether cells could interact with each other. One study showed that in a coculture of primary mouse myogenic cells and primary fibroblasts, there is a possibility of pluripotent fibroblast reprograming to myoblast (Melone *et al.*, 2000). While, coculturing adult fibroblasts with primary myogenic cells does not affect cellular origin and cells could affect growth of each other. Our coculture model is similar to models that have been used before. Ricotti et al reported on an NHDFC/C2C12 coculture model with engineered free-standing polyacrilamide gel, C2C12 differentiation, alignment towards one direction and expression of differentiation gene(actin and myosin) were improved (Ricotti *et al.*, 2013).

One other concern about coculturing adult fibroblast with C2C12 in a mixture is density-dependent contact inhibition of fibroblast on myoblast (Melone *et al.*, 2000). To avoid this, fibroblast were treated with mitomycin at 50% confluency to prevent

mitotic activity. Mitomycin treatment help us to save fibroblast-dependent ECM structure around C2C12 layer without fibroblast become overconfluent in the cell culture dish.

We used C2C12 cells as the muscle cell source. Few studies suggest that these cells could behave differently from primary muscle cells (Zou *et al.*, 2008). While collagen VI deposition was not detected in primary mouse muscle cells, C2C12 cells show evidence of collagen VI production in *in vitro* cultures (Zou *et al.*, 2008). Furthermore, collagen VI expression increased during C2C12 differentiation. Given the fact that we identify almost the same pattern of immunofluorescence staining for collagen XII in fibroblasts and the same localization in previous chapter, we hypothesize collagen XII might be secreted from C2C12 and affect their differentiation in the same way. However, we suggest expression and localization of collagen XII by C2C12 cells to be investigated through immunofluorescence staining.

We recognized coculturing C2C12 with human fibroblast in a mixture with direct contact between fibroblast and C2C12 increased C2C12 differentiation culturing on top of control fibroblast compares to C2C12 alone. While fibroblasts of affected individuals (BM5, BM1`) and DMD fibroblast did not increase C2C12 differentiation compare to C2C12 alone. Result of C2C12/NHDFC coculturing emphasize on previous findings (Ricotti *et al.*, 2013). These findings as indicated in the introduction show primary cardiomyocytes, when cocultured with fibroblasts, present better differentiation rate, improved alignment and higher expression of myoblast differentiation indicators(Pax7, Myo D) (Mann *et al.*, 2011), while mutant collagen XII fibroblast and DMD fibroblast do not influence C2C12 differentiation. An inhibitory paracrine effect on primary myoblast when cocultured with DMD fibroblast in a mixture was reported before. Overall, these findings show unlike NHDFCs mutant collagen XII fibroblast and DMD fibroblast did not give any physical or mechanical stimuli to C2C12 to increase differentiation. These findings suggest a possible role for collagen XII mutant fibroblast to prevent myoblast growth and differentiation in the affected individuals. However, the result of this *in vitro* experiment should be interpreted with caution for *in vivo* conditions.

We observed coculturing C2C12 with human fibroblasts without contact (inserted wells) does not decrease or increase C2C12s` differentiation in all fibroblast groups (mutant and control) compare to C2C12 alone. This result might echo the fact that C2C12 cells could secrete the required collagen molecules for differentiation process independently. However, production of collagen XII in C2C12 cells with appropriate antibody needs to be detected in future. Furthermore, this result could suggest the effect of NHDFC on C2C12 differentiation is not applicable through chemical stimuli alone. A physical or mechanical stimuli with or without chemical stimuli is required to induce higher differentiation rate in C2C12 cells.

We obtained an average differentiation rate 0.25% for C2C12s alone with a standard cell growth and differentiation culture. This finding is in accordance with other studies which culture C2C12 alone and use manual method of counting for identifying C2C12 differentiation.

The present study evaluates 2 major cell types of fibroblast and C2C12. However, in a more prospective approach the role of other ECM cells like endothelia cell in mutant and normal individuals should be evaluated. Another caveat is that we are focusing on isolated cell types in an *in vitro* situation. One of these cell types might behave differently from one in muscle tissue. Moreover, there is an open possibility that in an *in vivo* situation and different histologic context other stimuli and factors cover the role of mutant collagen XII molecules.

Findings of this research highlight possible interaction between fibroblast and myoblast during myoblast differentiation. In addition, it emphasizes the role of intact extracellular matrix and fibroblast component in myoblast differentiation. Mutant collagen XII secreted from affected individuals` fibroblast could destroy the appropriate histological context of muscle differentiation. It seems the possibility of this influence applied through direct contact is higher than being a paracrine effect. However, more studies on interaction between collagen XII and myoblast are required. One possible future work would be to investigate the localization of collagen XII in C2C12 cells to identify whether C2C12 interact with collagen XII directly.

Chapter 6 General discussion and future directions

Collagen VI-related myopathies are a group of heterogenic and clinically diverse neuromuscular disorders. The clinical presentations of collagen VI-related myopathy come as a spectrum with Ullrich congenital muscular dystrophy (UCMD) (MIM: 254090) at the severe end and Bethlem myopathy (BM) (MIM: 158810) at the milder end of this spectrum. In addition, an intermediate phenotype and limb-girdle muscular dystrophy (LGMD) phenotypes have been included into the collagen VI-related myopathy phenotypic spectrum (Bonnemann, 2011). Progressive muscle weakness with connective tissue involvement is a unifying feature of collagen VI-related myopathies. Connective tissue involvement includes distal joint contractures, joint hyperlaxity, skeletal deformities such as kyphoscolios is and skin involvements like keloid formation and hyperkeratosis pilaris (Bushby *et al.*, 2014). With a prevalence of 0.13 in 100,000 for UCMD and 0.77 in 100,000 for BM, these disorders are considered as rare neuromuscular disorders. However, the number of reported cases is increasing (Clement *et al.*, 2012).

Collagen VI-related myopathies in their severe forms are often inherited in autosomal recessive traits, while autosomal a dominant mode of inheritance is frequently found in milder phenotypes (Bonnemann *et al.*, 2011). In addition, severe forms of collagen VI-related myopathies can be caused by dominant *de novo* mutations (Mercuri *et al.*, 2002), and there are also more recent reports of recessive BM (Gualandi *et al.*, 2009).In 1996 (Jobsis *et al.*, 1996) in a large pedigree of Bethlem myopathy and in 2000 (Camacho Vanegas *et al.*, 2001) in Ullrich congenital muscular dystrophy individuals, mutations in *COL6A1* and *COL6A2* genes were reported to be disease-causing. Since then, several mutations in three distinct *COL6A* genes (*COL6A1*, *COL6A2* and *COL6A3*) have been identified in collagen VI-related myopathy patients. However, the underlying cause for several affected individuals with phenotypes highly reminiscent of collagen VI-related myopathy not bearing mutations in *COL6A* genes remains to be elucidated (Bonnemann, 2011).

Collagen VI is a beaded-filament forming member of collagen family. This heterotrimeric molecule is widely expressed in connective tissue in different organs

and plays a role as adhering, signalling and apoptotic element. Collagen VI' distinct role in neuromuscular connective tissue is not fully deciphered yet, however it is suggested it might affect regeneration, repair and autophagic pathways in muscular tissues (Bonnemann, 2011; Halper and Kjaer, 2014). Immunohistochemical studies demonstrate in muscle biopsy of collagen VI affected individuals, that collagen VI is not attached to basement membrane markers like perlecan or laminin ɣ (Bonnemann, 2011). Thus, dual labelling of collagen VI with basement membrane proteins such as perlecan and laminin is a robust but not gold standard method to predict collagen VI-related disorders. In addition to clinical presentation and examinations, other laboratory and paraclinical tools like Creatine kinase measurement, magnetic resonance imaging (MRI), immunofluorescence staining of dermal fibroblast in affected individuals for collagen VI and muscle biopsy findings are beneficial in distinguishing collagen VI-related myopathies from other hereditary neuromuscular disorders. However, full sequencing analysis of all three *COL6A* genes is currently the gold standard for diagnosis (Bonnemann *et al.*, 2011).

Diverse clinical features of collagen VI-related myopathy phenotypes are a reason for the large overlap with other neuromuscular disorders. Congenital muscular dystrophy, limb-girdle muscular dystrophy, merosin-deficient myopathy, Emery-Dreifuss muscular dystrophy, dystroglycanopathies and non-muscular disorders of connective tissue such as Ehlers-Danlos syndrome are among the differential diagnosis for collagen VI-related myopathies. This issue in combination with the genetic heterogeneity makes the diagnosis of collagen VI-related myopathy diagnosis challenging (Bushby *et al.*, 2014).

Several cases of "collagen VI-related myopathy -like" phenotype with no mutation in the collagen VI genes have been reported (Merlini *et al.*, 2008b; Bovolenta *et al.*, 2010). It has been reported that in approximately 75%-79% of UCMDs and 60-65% of BMs the genetic cause is identified by full sequencing of three *COL6A* genes (Bovolenta *et al.*, 2010). It is estimated that around 40%-50% of patients with phenotypes reminiscent or overlapping to collagen VI-related myopathy at the Newcastle centre for neuromuscular disorders are not caused by *COL6A* gene mutations (Debbie Hicks, personal communication).

In this study I investigated a group of 24 affected individuals of collagen VI-related myopathy phenotype (predominantly Bethlem-like) without *COL6A1-3* mutations. Whole-exome sequencing from five affected individuals of this group who are homogenous in clinical presentations generated a list of genetic variants. After excluding non-sense and polymorphic variants (MAF, >1%) based on *in silico* analysis, three to five variants of interest in each pedigree remained for further investigation. These variants were confirmed in the corresponding pedigrees by Sanger sequencing and pattern of inheritance for each of the variants was investigated. Two missense mutations in two pedigrees of affected individuals in the gene *COL12A1* were identified to segregate with expected mode of inheritance. *Col12A1*: c.C5893T:p.Arg1965Cys is a missense mutation which segregated in a pedigree of three affected individuals with autosomal dominant pattern of inheritance. This mutation lead to substitution of one arginine with a cysteine residue in the fibronectin type III (FN III) domain of the protein. The cysteine residue may be involved with disulfide bond formation in collagen XII structure (Halper and Kjaer, 2014). These bonds are necessary for stabilizing the collagen XII monomer. The c.G8357A: p.Gly2786Asp is other missense mutation which segregated in a trio with autosomal dominant pattern. This mutation leads to remove a glycine residue from GLY-X-Y motif in collagen XII α chain. GLY-X-Y motifs form a highly conserved region which is important in collagen α chains assembly to dimers and tetramers. In collagen VI, these mutations have proven to insert a kink in collagen VI fibrils. This kinked fibril could bond to other fibrils to make collagen VI tetramers and secreted to ECM. Thus, they are acting as dominant negative mutations (Lamande *et al.*, 2002). Previous findings showed patients with GLY-X-Y motifs substitution could represent typical Bethlem symptoms, while some reports finds sporadic mild Bethlem-like phenotype in patients with GLY-X-Y motifs substitution (Bonnemann, 2011). Autosomal-dominant substitutions in Gly-X-Y motifs lead to variable molecular and clinical consequences and depend on the exact sequence context in which they occur (Bonnemann *et al.*, 2011). When the mutated collagen alpha chains will participate in trimer assembly but subsequent folding of the triple-helical domain will be impaired, a dominant negative effect is exerted. Most deleterious are those missense changes that introduce residues with bulkier side chains. This was the case for BM2 with the replacement of glycine with aspartic acid (Long *et al.*, 1993;

Lamande *et al.*, 2002). The effect of the triple-helical domain misfolding is further augmented by excessive posttranslational modification, since the posttranslational reactions are normally terminated by folding of the protein into the triple helical conformation (Long *et al.*, 1993). Discovery of a splice variant *COL12A1* mutation potentially acting in dominant *de novo* fashion. The g:75814922-75815008del: c.8345-8432del:p.2727-2755del from whole exome sequencing of an affected individual with undiagnosed neuromuscular phenotypes, expand the clinical spectrum of collagen XII mutant patients. The mutation leads to in-frame deletion of exon 53 which leads to removing 27 amino acids from primary structure.

Immunofluorescence staining of dermal fibroblast in all affected individuals revealed decrease in collagen XII signals. In contrast, immunoblotting for cells from the patients with two missense mutation did not demonstrate any changes. Thus, in these patients collagen fibrils are secreted to the extracellular matrix but are unable to interact with other binding partners and to assemble in the ECM. In these patients immunofluorescence staining for two main collagen XII binding partner, collagen IV and tenascin reveals variations (Hicks *et al.*, 2014). Immunofluorescence staining for splice variant mutation demonstrated an absence of collagen XII in extracellular matrix structure and collagen XII decreased in immunoblotting of cell extract and media. Thus, mutant collagen XII in this patient may affect collagen fibril assembly and secretion to extracellular matrix leading to unstable protein. Laboratory findings and *in silico* analysis for *COL12A1* mutant patient suggested a misfolded protein and the clinical presentation of these patients shows some diversity. Lipodystrophy is a novel sign identified in one affected individual with the splice variant mutation, and might be unrelated to the myopathy. However, there are other myopathies that inherited with lipodystrophy (Bushby *et al.*, 2014). A panel of twelve known genes in lipodystrophy has been checked in exome data to identify any pathogenic mutation for lipodystrophy but no variants were discovered.

Lipodystrophy may result from activation of endoplasmic reticulum stress pathway. Misfolded protein which is trapped in endoplasmic reticulum is a source for cell stress. However, further investigations to confirm endoplasmic reticulum stress by inhibiting endoplasmic reticulum associated protein degradation (ERAD) pathway and apoptotic assay should be carried out to confirm this mechanism (Hughes *et al.*,

2017). Given the fact that affected individuals with *COL12A1* mutations who we discovered in our cohort represent diverse phenotypes and the number of collagen XII-related myopathy is yet limited, it is difficult to comment whether lipodystrophy could be mentioned in the phenotypic spectrum of collagen XII0related myopathy.

We identified overlapping symptoms of joint hyperlaxity and mild muscle weakness in all patients. However, muscle weakness was variable from mild to moderate in different patients. In addition, while for missplicing and glycine substitution mutations family, delayed motor milestones and skeletal deformity was discovered at birth, in the family with asparagine substitution the symptoms started in adulthood. This phenotypic diversity was reported in other investigation. Zou et al, found *COL12A1* mutations in one patient with severe muscle weakness and wheelchair dependency but for the other affected individual moderate muscle weakness was reported (Zou *et al.*, 2014).

Collagen XII is a member of fibril associated interrupted triple helix collagen (FACITs). This protein interacts with other extracellular matrix collagenous and non-collagenous components to stabilize collagen fibrils and control fibril growth during fibrillogenesis (Chiquet *et al.*, 2014). Given the fact that fibril growth is the result of increasing diameter and length of fibrils, we measured the diameter of collagen fibrils in extracellular matrix surrounding cultured fibroblast and after *in vitro* fibrillogenesis of extracted collagen from cultured fibroblasts. Statistical analysis reveals *COL12A1*mutations do not influence fibril diameter. However, in comparison to our *in vitro* assay *in vivo* fibrillogenesis may be a more complicated and multi-step process. Apart from fibroblast, other connective tissue cells like myofibres and environmental factors such as soluble water in ECM could affect this process (Koch *et al.*, 1995). Since we are unable to fully reflect *in vivo* conditions during our experiment this result may not reflect the composition and morphology of collagen fibers condition in human muscle. Therefore, a better understanding of collagen XII role in ECM may require additional experimental strategies to address challenges in studying collagen fibrillogenesis including genetically modified mouse models (Mienaltowski and Birk, 2014a).

While collagen XII is expressed in muscle tissue, its distinct role in muscle is unknown. The third part of our research focussed on the relationship between collagen XII and myofibre regeneration. Collagen VI studies revealed that collagen VI could affect myofibre differentiation and its expression increased during the myogenic cell differentiation (Zou *et al.*, 2008). Our adult fibroblast\C2C12 coculture system assesses the influence of mutant and normal fibroblast on C2C12 differentiation. Fibroblast / C2C12 cocultures were carried out by mixing cells with direct contact and growing cells in separated wells while they were fed with the same media but without direct contact. Mitomycin was used to inhibit mitotic activity of fibroblasts which could result in activation of apoptotic pathway. Statistical analysis demonstrated that direct contact with normal fibroblasts increases the differentiation rate in muscle cells (C2C12) significantly, but direct contact with mutant fibroblast did not increase the differentiation rate. In addition, coculture without direct contact did not further enhance C2C12 differentiation (Cooper *et al.*, 2004).

These findings confirm the importance of mechanical properties of ECM surrounding myocytes. The reduced differentiation rate in collagen XII mutant cells as well as Duchenne muscular dystrophy cells underlines the importance of intact ECM in myoblast differentiation.

One limitation for this study lies in the use of C2C12 cells which are of mouse origin and immortalised. It has been shown C2C12 cells act differently from primary myogenic cells in several assays (Zou *et al.*, 2008). The result of coculturing primary cell line for *in vivo* system must be interpreted with caution (Zou *et al.*, 2008).

Overall, we identified three mutations in the gene *COL12A1* in three pedigrees with six affected individuals. Immunofluorescence studies demonstrate reduction or absence of collagen XII in cultured cells. Thus, secreted collagen XII homotrimers are unable assemble into the extracellular matrix. However, these mutations did not affect collagen XII fibrils diameter according to our *in vitro* fibrillogenesis and cultured fibroblast collagen fibrils visualization assay by electron microscopy. Fibroblast\ C2C12 coculture studies implicate biomechanical effects of the extracellular matrix on myogenic cell differentiation.

Future directions

Our research deciphers pathogenic variants for three of our Bethlem-like myopathy phenotypes without *COL6A* mutations, but did not uncover the underlying genetic cause in 20 similar patients. Identifying the genetic-cause is important to determine possible therapeutic approaches and genetic counselling. Our exome sequencing approach is a valid and robust method to discover small, exonic variants in *COL6A1*, *COL6A2* and *COL6A3* genes but is unable to detect deep intronic mutations which may affect splicing or larger or more complex rearrangements (Bonnemann *et al.*, 2011). Thus, analysing RNA from appropriate cells or tissues using reverse transcriptase PCR (RT-PCR) or RNA sequencing may be required to detect identify aberrant splicing. Dose sensitive methods like multiplex ligation-dependent probe amplification (MLPA), genomic single nucleotide polymorphism array, or whole genome sequencing are likely to uncover larger and more complex rearrangements, but the interpretation may be time-consuming and challenging (Bonnemann, 2011).

Investigating collagen fibrils` diameter via preparation of intact extracellular matrix neighbouring cultured fibroblast and *in vitro* fibrillogenesis experiment with concentrated collagen extracted from cultured fibroblast are two methods to identify extracellular matrix ultrastructure and collagen XII mutation`s effect on this structure. These efforts do not show any variation between control samples and affected individuals. However, FACITs are regulators of collagen fibrillogenesis by mediating fibrils diameter and length increase (Mienaltowski and Birk, 2014b). Investigating length of collagen fibrils from skin biopsies by advanced electron microscopy techniques may be the next step for this line of investigation.

Altered biomechanical features in ECM of cultured fibroblast from affected individuals was observed in our coculture experiment, which was also found for Duchenne muscular dystrophy`s cultured fibroblasts.. In order to evaluate the distinct role of collagen XII in myogenic cells differentiation I would suggest to examine the interaction through immunofluorescence staining for collagen XII in C2C12 cultures. Further, quantitative PCR methods could be applied to check the level of collagen XII expression during myogenic cells differentiation.

Inhibiting endoplasmic reticulum degradation (ERAD) pathway in collagen XII mutant fibroblast is helpful to determine the effect of misfolded proteins on cells. This

experiment could determine whether the endoplasmic reticulum stress is involved in pathogenesis of these mutations. ERAD pathway activation could happen in all mutant individuals. For examples, in BM2 an Arginine substitution could lead to an unpaired cysteine which is in the middle of the unassembled branches and may change the homotrimer conformation. The unpaired cysteine residues are a substructure that triggers the recognition of misfolded or mutated proteins, along with exposed hydrophobic regions and immature glycans. When folding or assembly intermediates expose, unpaired cysteines, ER resident chaperones or oxidoreductases interact with them, and as a consequence, they are retained in the ER or retrieved from the Golgi. This process could activate the unfolded protein response (UPR) pathway. The underlying mechanism for this retention is unknown but residues could trap in endoplasmic reticulum and immature particles could form aggregates which could not exit from the ER in vesicle form. This aggregates then lead to dilated, swollen ER (Sitia and Braakman, 2003). In addition, constant supply of misfolded proteins due to genetic mutation which leads to activation of protein response pathway could initiate chronic ER stress, which in turn induces the cell to activate apoptosis (Shen *et al.*, 2004; Lai *et al.*, 2007). BM2, BM5and BM1`have mutations, which result in an absence or non-assembly of collagen XII in the ECM. UPR array which was done by Debbie Hicks showed an upregulation of UPR pathway in BM2 patients. Also, evidence of dilated ER by electron microscopy was seen in BM5 skin biopsies (Hicks *et al.*, 2014). These results provided proofs for activation of ERAD pathway in collagen XII mutant patients. Further investigation of ERAD pathway activation in BM1` individual who showed high reduction of collagen XII in ECM will be of benefit to provide more evidence for precise pathomechanisms acting in this family. Endoplasmic reticulum activation pathway is a proapoptotic mechanism. It is interesting to investigate whether apoptosis pathway is activated or not.

As precise pathomechanisms behind collagen XII mutant individuals is not deciphered, it is difficult to mention a distinct therapeutic drug in these individuals. However, according to our experience to follow collagen XII mutant individuals in MRC centre for neuromuscular diseases, palliative care could be of beneficial in these patients. BM5 individual is benefiting from walking aid. He also used a special

chair with higher sitting surface to reduce difficulties when tries to stand up. Another individual BM1` benefitting from surgery to treat recurrent jaw dislocation. The non-pharmacological interventions could have an important place in collagen XII individuals. Like other collagen muscular dystrophies disease-specific therapeutic modalities should be considered in these patients.

As we have not deciphered collagen XII pathomechanisms, potential and under evaluation therapies which has been considered in collagen VI-related myopathy such as anti-fibrotic therapy would not recommended in this stage for collagen XII patients.

Overall, we identified *COL12A1* as a novel disease-causing gene for Bethlem-like overlapping phenotype. Our immunohistochemical investigations revealed misfolding in collagen XII mutant protein. However, structural defect did not affect hierarchical structure of ECM in *COL12A1* mutant individuals. *In Vitro* analysis demonstrated that mutant collagen XII fibroblasts could not contribute in C2C12 differentiation as healthy donors.

Appendix A: collagen variants primers:

Col13A1,exon13,fwd	Gacttttggcatcgatctgaat	22	60.46	232
Col13A1,exon13,rev	Agagaggttctggctttgtctg	22	60.05	232
Col2A1,exon51,fwd	Cttttgtgtctgtgcctgtctg	22	60.92	294
Col2A1,exon51,rev	Agagtttgaggagccatctctg	22	60.02	294
Col6A1,exon14,fwd	Agttgattggcctcagtttacc	22	59.53	195
Col6A1,exon14,rev	gacagaagtcaaaacggtccac	22	60.95	195
Col12A1,exon41,fwd	gactaaatgtcagcactgaaaagg	24	59.37	242
Col12A1,exon41,rev	ttggcaaaatgtagaaaagattca	24	60.01	242
Col6A1,exon9,fwd	Gtcttttctcagtggtggcttt	22	59.79	354
Col6A1,exon9,rev	Atgcctctgtgagaccagtcc	21	61.66	354
Col6A2,exon 26,fwd	CTTCAAGGAGGCTGTCAAGAAC	22	60.42	468
Col6A2,exon 26,rev	Ctgcacattcatccctcaggt	21	60.03	468
Col19A1,exon28,fwd	Tcacttggataatttatggacattc	25	58.40	297
Col19A1,exon28,fwd	aatgggggagggtagataaaga	22	60.04	297
Col12A1,exon20,fwd	tggaaaagtgatacaaaagcttga	24	60.16	288

Col12A1,exon20,rev	aggctatcaaacgtgacacaaa	22	59.68	288
col6a,bm1,fwd	GAAGACACGCCAACACTAAGG	21	59.8	308
col6a3,bm1,rev	GGACGCTATCTTTCCAACCTC	21	59.8	308
col6a3,bm2,fwd	CAGGCTAGCGTGTTCTCATTC	21	59.8	268
col6a3,bm2,rev	Tcccacacagatggtgagaag	21	57.3	268
col22a1,bm3,fwd	Tgtcctatgtccgtttctgc	20	61.8	272
col22a1,bm3,rev	CTATGGGTCCAGAGGGTCCTT	21	55.9	272
col12a1bm3,fwd	GCCTTTGTTGGAGAAATGACA	21	55.9	284
col12a1,bm3,rev	Agcctgcctacataatgctga	21	57.9	284
col21a1,bm3,fwd	TCAAGAAAATTTGGGATTTATGG	23	53.5	302
col21a1,bm3,rev	Tatagccctcttttccccaac	21	57.9	302
col4a1,bm3,fwd	Tgtctttgtccagcttttgct	21	55.9	362
col4a1,bm3,rev	CATGTTCATTGGCATCAGAGC	21	57.9	362
gCOL6A6e7_f	AGCCTCAACAGAGTGCGAAT	20	57.3	314
gCOL6A6e7_R	TCAACATCATCATCCAATCCA	21	54.2	325

Col12A1,exon20,fwd	tggaaaagtgatacaaaagcttga	24	60.16	288
Col12A1,exon20,rev	Aggctatcaaacgtgacacaaa	22	59.68	288
Col12A1,exon41,fwd	gactaaatgtcagcactgaaaagg	24	59.37	242
Col12A1,exon41,rev	Ttggcaaaatgtagaaaagattca	24	60.01	242

Appendix B- Collgen XII affected individual's variant:

Ccol1212,fwd	CTGAGCTCTCCGCAGAAGG	19	61.0	882
Ccol1212,rev	ACGGTGATGGAGAAGTGGAC	21	59.4	882
Col12A1,exon41,fwd	gactaaatgtcagcactgaaaagg	24	59.37	242
Col12A1,exon41,rev	ttggcaaaatgtagaaaagattca	24	60.01	242
Col12A1,exon20,fwd	tggaaaagtgatacaaaagcttga	24	60.16	288
Col12A1,exon20,rev	aggctatcaaacgtgacacaaa	22	59.68	288

Appendix C- cDNA Collagen XII primer pairs:

Ccol12-1fwd	CCTCTCCGACCCTTTGAGC	19	61.0	924

Ccol12-1,rev	CTTCTGCTCTTCGCCTTCTCT	21	60.0	924
Ccol12-2fwd	AGACGTTCGTAGCCCACTGAG	21	61.8	814
Ccol12-2,rev	CATCATCTTCAGCACCTGGA	20	57.3	814
Ccol12-3fwd	AGACGTTCGTAGCCCACTGAG	21	60.5	764
Ccol12-3,rev	CCCAAAATTTCTTTCTGTTTATTCA	25	54.8	764
Ccol12-4,fwd	CTTCTGCTCTTCGCCTTC TCT	21	59.8	832
Ccol12-4,rev	CCCAAAATTTCTTTCTGTTTATTCA	25	54.8	832
Ccol12-5,fwd	CTTTTCCCTCCATGGTTTCTC	21	57.9	818
Ccol12-5,rev	TTTCCAGGATTGGAGGAAAA	20	53.2	818
Ccol12-6,fwd	GAAGGCCCAGTACTCAAGGTC	21	61.8	908
Ccol12-6,rev	CACTACTGGGAGGTTGACGTG	21	61.8	908
Ccol12-7,fwd	GCCCTAAGAAGCAGGCAGTAA	21	59.8	845
Ccol12-7,rev	GAACTGCAGTGCGGTGTTG	19	58.8	845
Ccol12-8,fwd	TTGAGGGGACTTGTGGTTTG	20	57.3	854
Ccol12-8,rev	TGGACGTCTTGAGCAAATCTT	20	55.9	854

Ccol12-9,fwd	CATCCGAGTGGTCCTGAGAG	20	61.4	835
Ccol12-9,rev	ACGGTGATGGAGAAGTGGAC	21	59.4	835
Ccol1210,fwd	CCCAAAATTTCTTTCTGTTTATTCA	25	54.8	746
Ccol1210,rev	CCTCTCCGACCCTTTGAGC	19	61.0	746
Ccol1211fwd	GGAACCCTAGCCCGAGAC	18	60.5	846
Ccol1211,rev	TGGACGTCTTGAGCAAATCTT	20	55.9	846
Ccol1212,fwd	CTGAGCTCTCCGCAGAAGG	19	61.0	882
Ccol1212,rev	ACGGTGATGGAGAAGTG	21	59.4	882

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